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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> Physiologic induced drug resistance to topoisomerase II inhibitors was studied in EMT6 mouse mammary tumor cell lines created under the award. EMT6 cells transfected with ponasterone A inducible plasmids containing either the p65 subunit of NFkB or the p50 subunit of NFkB or the non-phosphorylatable mutant of IkB were assessed for NFkB activation and drug resistance in the presence or absence of stress. Expression of the p65 or p50 subunits resulted in drug resistance to etoposide and doxorubicin. Expression of the IkB mutant which prevents the activation of NFkB blocked the development of stress induced drug resistance. These data demonstrate that NFkB is necessary and sufficient for the development of stress induced resistance.			
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Introduction

The success of treatment for breast cancer is often limited by the development of drug resistance. We have described a unique type of resistance induced by the physiological factors at play within solid tumors. Solid tumors, due to inadequate vascularization contain microenvironmental regions that are hypoxic and thereby subject the neoplastic cells in these regions to cellular stress. The cellular response to this stress then determines cellular susceptibility to therapy. EMT6 mouse mammary tumor cells treated with hypoxia (paper in appendix) or the chemical stress agent brefedin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin et al, 1998, Brandes et al, 2001). Furthermore, data obtained through the present grant show that this chemically- and physiologic-induced resistance is mediated in large part by the nuclear transcription factor NF- κ B. The overall scope of the work is to determine if NF- κ B is the mediator of physiologic induced resistance and whether agents which alter NF- κ B activation can alter the effectiveness of topoisomerase II type drugs.

Body

Tasks 1- 3 from Year 1 were to insert the NF- κ B p65 plasmid and then isolate, verify expression and determine drug resistance in a high and low expressor cell line. While we were able to obtain cell lines transfected with the NF- κ B plasmid for p65 or the alternative subunit, p50, the actual expression of p65 or p50 was not elevated and in fact appeared to be lower. We modified our plans to address this issue. This year, drawing on our experience with the I κ B α M cell line, we have inserted both the p65 and p50 subunits into the ponasterone inducible vector system (for discussion of I κ B α M results in the inducible vector system please see Brandes et al, Molecular Pharm., in press, 2001, copy found in the appendix). Our previous data using this system has demonstrated that ponasterone A did not activate NF- κ B in EMT6 cells, did not interfere with stress induced NF- κ B activation, and was not toxic to the cells. Both vector p65 and p50 containing cells and empty vector cells were made for these experiments. Briefly, we verified that ponasterone induced expression of p65 and p50 using western blot analysis for protein; showed that the induction of p65 and p50 in the cells led to enhanced NF- κ B activation as measured by luciferase assays, and showed that p65 or p50 expressing cells were resistant to etoposide and doxorubicin, topoisomerase II inhibitors. Detailed results of these experiments are found in the reprint of the poster and talk presented on this work at the 92nd Annual meeting of the American Association for Cancer Research and can be found in the appendix.

We also began looking at the effects of p65 or p50 expression on stress induced responses. In all three transfected cell lines (EMT6-vct, EMT6-p65, EMT6-p50), BFA was able to induce not only NF- κ B activation but also drug resistance (see Figure A

and Figure B below). However, when either p65 or p50 subunits were expressed, stress-induced activation of NF- κ B was comparable to the unstressed, basal level of NF- κ B activation and drug resistance was lower than in VCT (empty vector) controls.. This abrogation of stress-induced activation was reflected in the lack of stress induced drug resistance during p65 or p50 expression. This type of result was surprising but has been seen in other stress pathways. For example, in the UPR pathway, mediated by unfolded protein, GRP78 (BiP) induction by stress is abrogated if in fact GRP78 is overexpressed (Dorner et al, 1987).

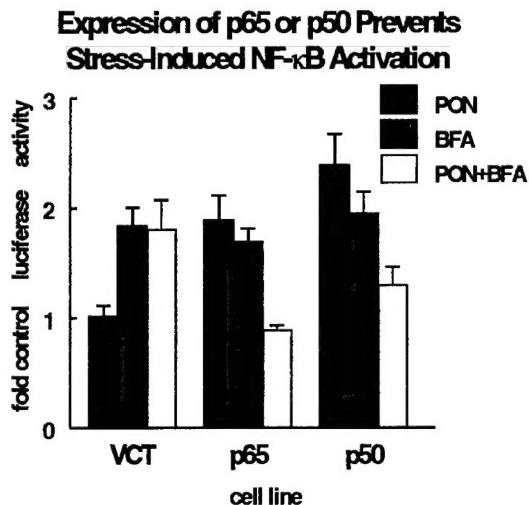


Figure A. Expression of p65 or p50 prevents stress induced NF- κ B activation. Cells containing empty vector (VCT) or p65 or p50 were treated with Ponasterone A only (PON-blue bars), BFA (red bars) or the combination PON+BFA (white bars). NF- κ B dependent activation of luciferase activity was measured. Treatment with ponasterone or with BFA resulted in increased luciferase activity. Treatment with ponasterone and BFA in cell lines containing p65 or p50 plasmids led to lower luciferase activity compared with VCT only controls.

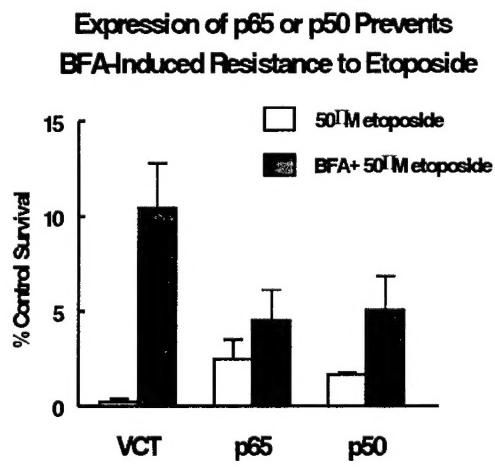


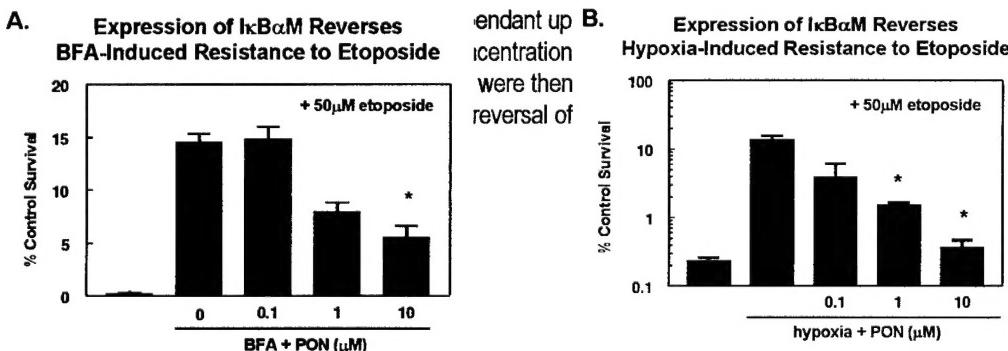
Figure B. Expression of p65 or p50 prevents BFA-induced resistance to etoposide. Cells were treated with or without BFA for 30 min as a stress after pretreatment for 24h with ponasterone A to induce p65 or p50 expression. Eight hours after BFA stress treatment cells were treated with 50 μ M etoposide for 1 h and then assayed for viability using colony formation. Resistance to etoposide with stress was lower in p65 or p50 expressing cells than in empty vector containing cells.

Additional investigation is required to determine the mechanism behind this finding.

It may be that NF- κ B activation by overexpressing either p65 or p50 may have simply induced sufficient increases in I κ B content in these cells such that stress could not induce NF- κ B activation as efficiently. Indeed, our preliminary expression array data suggests that in fact I κ B is induced during NF- κ B activation as expected. We have not performed any array expression studies after p65 or p50 overexpression.

Task 4 and 5 from Year 1 concerned comparative experiments using an I κ B α M which was not phosphorylatable and therefore could prevent the activation of NF- κ B. The completed results from these studies are delineated in our paper which is in press in **Molecular Pharmacology**. A copy of the page proofs are in the Appendix. In brief, we showed that I κ B α M expressing cells had similar sensitivities to etoposide as vector only expressing cells in the absence of stress; that stress would induce similar levels of drug resistance in both VCT and I κ B α M cells (non-induced); that in I κ B α M (induced) the cellular sensitivity to etoposide was similar to that of non-stressed cells and that biochemical measurements of NF- κ B activation correlated with the cellular toxicity data (expression of I κ B α M prevented stress-induced activation of NF- κ B). This was true whether the stress used in the experiments was BFA (brefeldin A), HYP (hypoxia) or OA (okadaic acid). We conclude from the data from Task 1-5 that stress induced activation of NF- κ B is the likely mechanism by which stress renders tumor cells resistant to drug induced toxicity.

In year 2, Tasks 1 and 2 were to compare drug resistance in transfected cell lines under conditions in which varying levels of expression were obtained. The original design was to isolate and use cloned with various levels of expression. To accomplish this with the inducible lines, we first attempted to determine if altering the concentration of inducing agent would alter the degree of resistance or the amount of resistance reversal that one would obtain. As seen below, our preliminary results with the I κ B α M expressing cell line suggests that this is the case.



In addition, we have begun similar studies with p65 and p50 expressing cell lines as

shown below in Figure D. The data also suggest that the level of resistance is dependent upon the concentration of ponasterone used for induction of the plasmid.

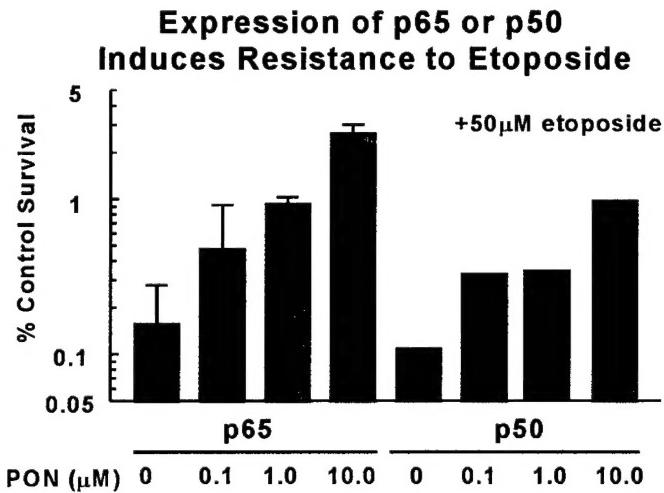


Figure D. Expression of p65 or p50 Induces Resistance to Etoposide. Cells were pretreated with varying concentrations of the plasmid inducing agent, Ponasterone (PON) for 24 h. The cells were then exposed to 50 μ M etoposide for 1 h and cytotoxicity was assessed by colony formation. Cellular sensitivity to etoposide was dependent upon the amount of inducing agent used.

Additional experiments will be required to complete the data set. In addition, to show that induced activity is dependent upon expression, both western blot experiments and functional assays of NF- κ B activity using the luciferase system will be required. We are in the process of performing these assays and studies.

Our background studies with the pharmacological inhibitor of NF- κ B activation, prostaglandin A₁,(PGA₁) have been submitted and accepted for publication in Oncology Research. These studies have demonstrated that stress treatment did not alter intracellular drug accumulation, topoisomerase II protein levels or inhibit topoisomerase II activity. Both hypoxia and BFA caused activation of NF- κ B. Pretreatment of the cells with PGA₁ inhibited stress-induced activation of NF- κ B and reversed BFA and hypoxia induced resistance. Resistance could be reversed when PGA₁ was given prior to or after the stress. These data imply that agents like PGA₁, which can abrogate the activation of NF- κ B could be useful adjuncts to enhance the clinical efficacy of topoisomerase II directed chemotherapeutics. A copy of this manuscript is included in the appendix.

We have begun to determine the downstream signaling pathways that are involved in mediating resistant phenotype. The rationale behind these experiments is that understanding the downstream signals may allow us to refine the target. Because NF- κ B is such a general transcription factor and plays a role in so many physiologic processes, the use of a general inhibitor may lead to a plethora of unintended effects and toxicities. Our hypothesis is that ER-stress via the ER overload pathway leads to the induction of a common subset of signals which mediate the resistance and that by comparing stresses and the reversal of stresses using expression arrays we may be able to focus in on a relatively small subset of genes. Our preliminary data which has not been fully analyzed suggests that this is the case. We have used the Affymetrix mouse expression array system to perform initial assessments. Using matrix cross analyses of hypoxia and BFA with or without induction of I κ BM induction, we can narrow the number of genes that represent the intersection of the two stresses to 40-60. In addition, initial classifications of the involved genes show that a wide variety of cell processes including cell cycle, energy metabolism, and apoptosis related genes may be involved. In addition, cursory inspection of the data show that expected gene products like I κ B are increased during stress (NF- κ B activation) and decreased during expression of I κ BM during stress (inhibition of NF- κ B activation). These data are shown, in part, in the poster presentation from the AACR meeting "Reversal of stress induced resistance of breast tumor cells to topoisomerase II inhibitors with inducible expression of a dominant negative mutant of I κ B α ". A copy of this poster can be found in the Appendix.

Task 6 from year 1 and Tasks 3 and 4 from year 2 concern the effects of NF- κ B inhibitors on *in vivo* tumors. Again this year, these studies have not begun. Although it was initially thought that the contamination of the mice from mouse hepatitis virus would be easily contained and eliminated, this has proven to be more difficult. The institution concluded that in order to maintain clean animals it would need to build a barrier facility. The facility is in the process of completion and should be turned over to the University by September. After a period of testing to ensure that the animals are maintained virus free, investigators should be able to begin using the facility. When the barrier facility becomes available, we will commence these studies. We feel strongly that it would be foolish to perform the studies on sick animals which would lead to invalid and potentially irrelevant results.

Recommended Changes to the Statement of Work for Year 3.

1. Use the inducible systems to assess effects of expression levels and to identify common genes. Use the inducible system to extend the work to human breast cell lines.
2. Perform animal tumor studies with PGA₁.

Key Research Accomplishments

- Determined that okadaic acid activated NF- κ B and resulted in drug resistance similar to BFA and hypoxia.
- Determined that induction of I κ B α by ponasterone could reverse NF- κ B activation and reversed drug resistance.
- Constructed the inducible p65 and p50 plasmids, pIND-p65 and p-IND-p50.
- Transfected the pIND -p65 and p50 plasmids into EMT6 cells and isolated hygromycin resistant clones.
- Demonstrated that ponasterone A induction of the p65 or p50 transfected cells resulted in the expression of the proteins and that the expression of p65 and p50 led to the development of drug resistance to etoposide and doxorubicin.
- Showed that induction of p65 or p50 cells led to increased NF- κ B activity as measured by elevation of luciferase.
- Demonstrated that induction of p65 or p50 subunits led to the inhibition of stress induced NF- κ B activation and inhibition of stress induced drug resistance.
- Used expression arrays to begin to dissect the common downstream pathways for NF- κ B mediated drug resistance.

Reportable Outcomes

Presentations:

L.M. Brandes, D.A. Stephan, S. R. Patierno, and K.A. Kennedy, Reversal of stress-induced resistance to topoisomerase II inhibitors with inducible expression of a dominant negative mutant of I κ B α , Proc. Amer. Assoc. for Cancer Res. 42: 813 (Abstract #4361), 2001.

L.M. Brandes, S.R. Patierno, and K.A. Kennedy, Inducible expression of the p65 and p50 NF- κ B subunits causes resistance to topoisomerase II inhibitors, Proc. Amer. Assoc. Cancer Res. 42: 122 (Abstract #665) 2001. *Note: This abstract was published but not presented due to a death in the presenting author's family.*

L.M. Brandes, S. R. Patierno, D.A. Stephan, and K.A. Kennedy, Stress-induced resistance of breast tumor cells to topoisomerase II inhibitors is mediated by NF- κ B activation, Annual meeting of the Washington Chapter of the Society for Experimental

Biology in Medicine, 2001.

Papers:

L. M Brandes, Z.P. Lin, S. R. Patierno, and K.A. Kennedy, Reversal of physiological stress-induced resistance to topoisomerase II inhibitors using an inducible phosphorylation site-deficient mutant of I κ B α , Molecular Pharm. 60: 1-9, 2001.

Y.C. Boller, L.M. Brandes, R. L. Russell, Z. P. Lin, S. R. Patierno, and K. A. Kennedy, Prostaglandin A₁ inhibits stress-induced NF- κ B activation and reverses resistance to topoisomerase II inhibitors, in press, Oncology Res., 2001.

Cell Lines:

Stable ecdysone inducible expression of p65 in the EMT6 mouse mammary tumor cell line- EMT6-p65.

Stable ecdysone inducible expression of p50 in the EMT6 mouse mammary tumor cell line- EMT6-p50.

Research opportunities applied for and or received on experiences/training supported by this award:

L.M. Brandes, Predoctoral Award, US Army BCRP, BC000486, Alterations in gene transcription by physiological stress: a mechanism for drug resistance through NF- κ B activation, 2001.

CONCLUSIONS

Our results show that NF- κ B activation by physiologic stress leads to resistance to topoisomerase II type drugs including etoposide, teniposide, and doxorubicin. Pharmacologic agents which interfere with NF- κ B activation can reverse resistance when given before, during or after stress. Genetic manipulation of the I κ B α subunit to block NF- κ B activation render the cells completely sensitive to drug during stress. Overexpression of either the p50 or p65 subunits of the transcription factor also leads to drug resistance. These data show that NF- κ B expression is sufficient and necessary to cause cells to become resistant to topoisomerase II inhibitors. Manipulation of tumor NF- κ B activation or manipulation of the downstream signaling events arising from NF- κ B activation should lead to altered responsiveness to topoisomerase II inhibitors.

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A. J. Dorner, L.C. Wasley, and R.J. Kaufman, Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells, EMBO Journal 11: 1563-1571, 1992.

Reversal of Physiological Stress-Induced Resistance to Topoisomerase II Inhibitors Using an Inducible Phosphorylation Site-Deficient Mutant of $\text{I}\kappa\text{B}\alpha$

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ABSTRACT

Physiological stress conditions associated with the tumor microenvironment play a role in resistance to anticancer therapy. In this study, treatment of EMT6 mouse mammary tumor cells with hypoxia or the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes the development of resistance to the topoisomerase II inhibitor etoposide. The mechanism of physiological stress-induced drug resistance may involve the activation of stress-responsive proteins and transcription factors. Our previous work shows that treatment with BFA or OA causes activation of the nuclear transcription factor NF- κ B. Pretreatment with the proteasome inhibitor carbobenzoyl-leucinyl-leucinyl-leucinal inhibits stress-induced NF- κ B activation and

reverses BFA-induced drug resistance. To test whether NF- κ B specifically mediates stress-induced drug resistance, an inducible phosphorylation site-deficient mutant of $\text{I}\kappa\text{B}\alpha$ ($\text{I}\kappa\text{B}\alpha\text{M}$, S32/36A) was introduced into EMT6 cells. In this study, we show that $\text{I}\kappa\text{B}\alpha\text{M}$ expression inhibits stress-induced NF- κ B activation and prevents BFA-, hypoxia-, and OA-induced resistance to etoposide. These results indicate that NF- κ B activation mediates both chemical and physiological drug resistance to etoposide. Furthermore, they imply that coadministration of agents that inhibit NF- κ B may enhance the efficacy of topoisomerase II inhibitors in clinical cancer chemotherapy.

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Solid tumors often have irregular and inadequate vascularization because of the uncontrolled cellular growth associated with tumor formation. Inadequate blood flow creates cell subpopulations within tumors that are hypoxic and/or glucose-deprived (Vaupel et al., 1989). These physiological stress conditions can result in tumor subpopulations with altered biochemical properties. Alterations such as decreased growth fraction or enhanced DNA repair can result in the development of intrinsic resistance against topoisomerase II-directed anticancer agents (Shen et al., 1987). Resistance to topoisomerase II inhibitors can also be induced by chemical stress agents that cause the inhibition of protein glycosylation, release of intracellular calcium stores, or disruption of endoplasmic reticulum (ER)-to-Golgi transport (Hughes et al., 1989; Lin et al., 1998). Taken together, these results suggest that physiological-based chemotherapeutic resistance may involve the induction of cellular stress pathways.

Under chemical or physiological stress conditions, ER function is often compromised because of the accumulation of normally folded proteins in the ER (Pahl and Baeuerle, 1997). High ER protein levels activate a cellular stress pathway known as the ER-overload response (EOR). In this pathway, ER protein overload causes the release of intracellular Ca^{2+} , formation of reactive oxygen intermediates, and activation of the nuclear transcription factor NF- κ B (Pahl and Baeuerle, 1997). Evidence now shows that physiological and chemical stress agents that result in drug resistance cause the activation of the EOR pathway and the transcription factor NF- κ B (Hughes et al., 1989; Pahl and Baeuerle, 1997; Lin et al., 1998). NF- κ B is a heterodimeric transcription factor usually composed of the p65 and p50 DNA-binding subunits (Urban et al., 1991). Under most circumstances, NF- κ B is in an inactive state, bound to an inhibitory protein, $\text{I}\kappa\text{B}$, in the cytosol. Three major isoforms of $\text{I}\kappa\text{B}$ have been identified, of which $\text{I}\kappa\text{B}\alpha$ is believed to be the predominant form (Tran et al., 1997). To activate NF- κ B, $\text{I}\kappa\text{B}\alpha$ is phosphorylated, ubiquitinated, and then degraded by proteasomes (Henkel et al., 1993). Point-mutation analysis has shown that $\text{I}\kappa\text{B}\alpha$ is specifically phosphorylated at two resi-

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ABBREVIATIONS: ER, endoplasmic reticulum; EOR, endoplasmic reticulum-overload response; NF- κ B, nuclear factor- κ B; BFA, brefeldin A; OA, okadaic acid; MG-132, carbobenzoyl-leucinyl-leucinal; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; $\text{I}\kappa\text{B}\alpha\text{M}$, S32/36A mutant inhibitory nuclear factor- κ B protein α ; VCT, ●●; TBST, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; ANOVA, analysis of variance; UPR, unfolded protein response.

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dues, serines 32 and 36, and phosphorylation site-deficient mutants are incapable of activating NF- κ B (DiDonato et al., 1996). Degradation of I κ B exposes a nuclear localization sequence that allows the translocation of NF- κ B into the nucleus, where it then binds to κ B motifs in promoter regions and directs the transcription of NF- κ B-sensitive genes (Harrag and Sun, 1999).

In addition to its role in cellular stress responses, NF- κ B activation is known to protect cells from apoptosis. NF- κ B activation suppresses the activation of caspase-8 through the regulation of tumor necrosis factor receptor-associated factor protein and inhibitor of apoptosis protein (Wang et al., 1998) and prevents cytochrome *c* release through activation of A1/Bfl-1, a Bcl-2 family member (Wang et al., 1999). Inhibition of NF- κ B activation with expression of a mutant I κ B α sensitizes tumor cells to apoptotic death by tumor necrosis factor, paclitaxel, and daunorubicin (Wang et al., 1996; Batra et al., 1999; Huang et al., 2000). Mutant I κ B α expression *in vivo* significantly reduces growth of head and neck squamous cell carcinoma (Duffey et al., 1999) and sensitizes chemoresistant tumors to the toxic effects of camptothecin (Cusack et al., 2000). These results suggest that physiological stress-induced NF- κ B activation may modulate the expression of apoptosis genes and that inhibition of NF- κ B activation may prevent stress-induced drug resistance.

We have shown that EMT6 mouse mammary tumor cells treated with the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes NF- κ B activation and resistance to the topoisomerase II inhibitor teniposide (Lin et al., 1998). Treatment with BFA disrupts protein transport from the ER to the Golgi apparatus and causes activation of the EOR response (Pahl and Baeuerle, 1997). OA treatment inhibits the PP1 and PP2A phosphatases, resulting in phosphorylation of I κ B and NF- κ B activation (Trevenin et al., 1990). We have also shown that pretreatment with the proteasome inhibitor MG-132 inhibits NF- κ B activation induced by BFA and reverses BFA-induced resistance to teniposide (Lin et al., 1998). In the present study, we show that BFA, hypoxia, and OA induce resistance to the clinically relevant topoisomerase II inhibitor etoposide. We tested whether specific inhibition of NF- κ B with the phosphorylation site-deficient mutant of I κ B α (I κ B α M, S32/36A) prevents stress-induced NF- κ B activation and reverses BFA, hypoxia-, and OA-induced resistance to etoposide. Our results show that NF- κ B is a key mediator of both chemical and physiological resistance to etoposide.

Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells, provided by Dr. Sara Rockwell (Yale University, New Haven, CT), were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25 μ g/ml gentamicin sulfate (Biofluids, Rockville, MD). Cells were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C and passaged every 3 to 4 days.

Reagents and Treatments. Brefeldin A (Sigma) was dissolved in 70% ethanol to a concentration of 10 mg/ml and stored at 4°C. For electrophoretic mobility shift assay (EMSA) and luciferase assays, cells were exposed to 10 μ g/ml BFA for 2 h and then incubated for 2 h in BFA-free media. For colony-forming assays, cells were exposed to BFA for 2 h and then incubated in BFA-free media for an additional

6 h. Okadaic acid (Calbiochem, La Jolla, CA) was kept at a concentration of 100 μ M in dimethyl sulfoxide and stored at -20°C. Cells were treated with 60 nM OA for 8 h in all experiments. Etoposide (Sigma) at 100 μ M in dimethyl sulfoxide was stored at -20°C. Cells were treated with 10 to 50 μ M etoposide 1 h before clonogenicity assay. Ponasterone A (Invitrogen) was rehydrated in 70% ethanol to a concentration of 10 mM and stored at -20°C. For all experiments, cells were treated with 10 μ M ponasterone A for 24 h to obtain maximal expression of I κ B α M. For all assays involving hypoxia, cells were grown in 75-cm² glass flasks for 48 h and then exposed to continuous hypoxia as described previously (Rockwell et al., 1982) for either 2 h (EMSA and luciferase assays) or 8 h (colony-forming assays). For EMSA and luciferase assays, the length of stress treatment was chosen to correspond with the time of maximal stress-induced NF- κ B activation as determined previously (Lin et al., 1998; data not shown).

Inducible I κ B α M Transfection. EMT6 cells were stably transfected with a phosphorylation site-deficient mutant of I κ B α (I κ B α M, S32/36A, provided by Dr. Michael Karin, University of California, San Diego), which also contains three hemagglutinin (HA) tags (DiDonato et al., 1996) or a control vector lacking I κ B α M (VCT) using the ecdysone-inducible expression system (Invitrogen). The I κ B α M gene was first ligated into the inducible plasmid pIND to create the pIND-I κ B α M plasmid. The other plasmid in the system, pVgRXR, encodes a modified ecdysone receptor and retinoid X receptor that dimerize in the presence of the inducing agent, ponasterone A, and binds response elements on the pIND plasmid. For transfection, EMT6 cells were seeded at a density of 3 to 4 \times 10⁴ cells/ml in 25-cm² flasks and were grown for 20 h. Cells were transfected for 1 h with 1 μ g of pIND or pIND-I κ B α M plasmid, 5 μ g of pVgRXR plasmid, and 36 μ l lipid transfection reagent (TransFast; Promega, Madison, WI) in 2.5 ml of serum-free Waymouth's media. Transfected cells were then seeded in 100 mm² tissue-culture dishes and treated with 400 μ g/ml hygromycin B (Invitrogen) to select for incorporation of the pIND vector. Selected clones were screened by Western blot and luciferase assay for inducible I κ B α M expression. Cell lines were maintained in Waymouth's media containing 400 μ g/ml hygromycin and grown in Waymouth's media without hygromycin for 40 h before experimentation.

Western Blot Analysis. Cells were seeded in 100-mm² dishes and grown for 18 h. After treatment with ponasterone A, total cell lysates were collected by homogenizing cell pellets in 100 μ l of 1× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, and 0.006% bromophenol blue). Protein lysate (20–100 μ g) was mixed with 2× SDS (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, and 2% β -mercaptoethanol), separated on a SDS-polyacrylamide gel (4% stacking gel, pH 6.8; 10% resolving gel, pH 8.8; 30:0.8 acrylamide/bisacrylamide), and transferred to a nitrocellulose membrane by electrophoresis. After transfer, the membrane was blocked in 1× TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) with 1% bovine serum albumin (BSA) and then probed with an anti-I κ B α antibody (C21; Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:1000 in 1× TBST with 1% BSA overnight at 4°C. The membrane was then washed with 1× TBST and incubated with an horseradish peroxidase-conjugated IgG anti-rabbit secondary antibody (1:10,000 dilution in 1× TBST with 1% BSA) for 1 h at room temperature. Immunoreactive bands were observed with enhanced chemiluminescent reagent (Pierce Chemical, Rockford, IL). After observation, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.67% β -mercaptoethanol) at 50°C for 30 min, washed in 1× TBST for 1 h, and probed again with anti-HA and anti-actin primary antibodies (Santa Cruz Biochemicals).

Transient Transfection and Luciferase Reporter Gene Assay. We obtained a luciferase reporter plasmid, pTk-(κ B)₆-Luc (provided by Dr. Heike Pahl, University Hospital, Freiburg, Germany), that contains six NF- κ B binding sites (κ B elements) upstream of a minimal thymidine kinase promoter (Bachelerie et al., 1991). Cells

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were seeded at a density of 3 to 4 $\times 10^4$ cells/ml in 60-mm² dishes or 25-cm² flasks and grown for 20 h. Cells were transfected with 2.5 ml of serum-free Waymouth's media containing 3 μ g of the luciferase reporter plasmid and 1 μ g of pcDNA3.1-*lacZ* (Invitrogen) in 12 μ l of reagent (TransFast; Promega). After drug treatments, cells were lysed for 15 min at room temperature in 400 μ l of reporter lysis buffer (Promega) and cleared of cell debris by centrifugation. For the luciferase assay, 100 μ l of luciferase assay reagent containing luciferol (Promega) was added to 20 μ l of cell lysate. Light emission was measured using a Beckman scintillation counter using the single-photon monitor mode over a 1-min interval. Cells were also assayed for *lacZ* expression to correct for differences in transfection efficiency. Cell lysate (100–150 μ l) was mixed with an equal amount of 2 \times assay buffer containing *o*-nitrophenyl- β -D-galactopyranoside (Promega) and incubated for 2 h at 37°C. Absorbance was measured at 420 nm was measured, and the relative β -galactosidase activity for each sample was used to normalize luciferase activities.

EMSA. EMSA was performed as described previously (Lin et al., 1998). Briefly, cells were seeded at a density of 3 to 4 $\times 10^4$ cells/ml in 150-mm² dishes or 150-cm² glass flasks. After drug treatment, cells were lysed in 100 μ l of lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) for 5 min at 4°C. Cell nuclei were separated by centrifugation at 5000 rpm for 5 min at 4°C, washed with 500 μ l of washing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride), and broken by three freeze-thaw cycles. To construct the probe, 3.5 pmol of oligonucleotide containing the NF- κ B consensus sequence (Promega) was incubated with 1 μ l of [γ -³²P]ATP (10 mCi/ml, 6000 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL), 5 units of T4 polynucleotide kinase (Promega), and 10 μ l of end-labeling buffer at 37°C for 1 h and then terminated with 90 μ l 1 \times Tris/NaCl/EDTA buffer (Sigma) and passed through a G-25 spin column (Worthington Biochemicals, Freehold, NJ). Nuclear protein extract (15–20 μ g) was incubated with 3 μ g of poly dI-dC and 0.035 pmol of radiolabeled oligonucleotide (100,000–200,000 cpm) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 0.5 mM dithiothreitol, and 4% glycerol) at room temperature for 20 min and separated on a nondenaturating 6% polyacrylamide gel (30:1 acrylamide/bisacrylamide, 0.5 \times Tris/borate/EDTA, and 2.5% glycerol). The resulting gel was transferred to filter paper, dried under vacuum pressure, and exposed to X-ray film.

Colony-Forming Assay. Cells were seeded in 25-cm² plastic flasks or 75-cm² glass flasks at a density of 3 to 4 $\times 10^4$ cells/ml and grown for 18 to 40 h before treatment. VCT and I κ B α M cells were treated with ponasterone A 24 h before colony assay, with stress 8 h before colony assay, and with 10 to 50 μ M etoposide 1 h before colony assay. After drug treatments, cells were harvested with trypsin and serially diluted in Waymouth's medium, as described previously (Lin et al., 1998). After 7 to 10 days, colonies were stained with 0.25% crystal violet and counted. For each treatment, the percentage of control cell survival was determined by dividing the cell survival of drug-treated cells by the cell survival of appropriate nontreated or solvent-treated cells.

Statistics. For colony-forming assays, the results shown are the average percentage of control survival \pm S.E.M. from three to five independent experiments, with three replicates per experiment. For luciferase assays, the results shown are the relative control luciferase activity \pm S.E.M. from three to five independent experiments. Statistically significant changes in these data were determined using one-way ANOVA with multiple comparisons performed using Bonferroni's test using $p < 0.05$ (Motulsky, 1995).

Results

Treatment with BFA, Hypoxia, and OA Induce Resistance to Etoposide. Chemical and physiological stress con-

ditions are known to activate ER stress pathways and induce resistance to topoisomerase II inhibitors (Hughes et al., 1989; Lin et al., 1998). We first determined the effect of the ER stress agents BFA, hypoxia, and OA on the clonogenic survival of etoposide-treated EMT6 cells. Cells were exposed to hypoxia for 8 h, 60 nM OA for 8 h, or 10 μ g/ml BFA for 2 h, followed by recovery in BFA-free media for 6 h. Etoposide at various concentrations was added during the last hour of stress treatment before analysis by colony-forming assay. Plating efficiencies were corrected for survival changes caused by treatment with BFA (plating efficiency = 110% of control), hypoxia (plating efficiency = 80% of control), or OA (plating efficiency = 38% of control). Figure 1 shows that pretreatment with BFA, hypoxia, or OA causes greatly enhanced cell survival in the presence of etoposide compared with nonstressed cells. These data suggest that the chemical and physiological conditions known to activate the EOR pathway induce resistance to etoposide.

Inducible I κ B α M Expression in EMT6 Cells. We and others have shown that chemical and physiological stress agents that cause ER stress lead to the activation of NF- κ B (Lin et al., 1998; Pahl and Baeuerle, 1997). Furthermore, inhibition of NF- κ B is known to enhance the toxicity of cancer therapeutics (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000) and reverse stress-induced drug resistance (Lin et al., 1998). Therefore, we hypothesized that BFA, hypoxia, and OA cause resistance to topoisomerase II inhibitors through the activation of NF- κ B. To test this hypothesis, we selected EMT6 cells transfected with either a phosphorylation site-deficient mutant of I κ B α (I κ B α M) or a control vector lacking I κ B α M (VCT) using the ecdysone-inducible expression system. After selection in hygromycin, we screened transfectants by using Western blot analysis for expression of I κ B α M after treatment with the inducing agent, ponasterone A.

The I κ B α M we obtained also contains three HA tags (DiDonato et al., 1996), which cause the mutant protein to migrate more slowly than wild-type I κ B α when analyzed by gel electrophoresis. Figure 2 shows that both VCT cells treated with ponasterone A and noninduced I κ B α M cells

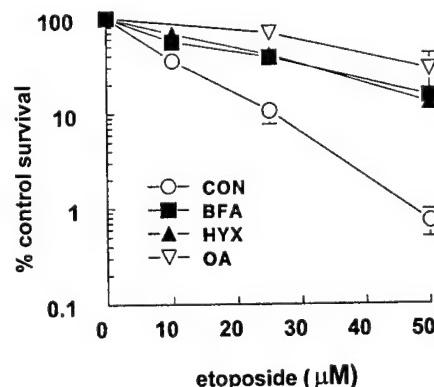


Fig. 1. Brefeldin A, hypoxia, and okadaic acid treatment cause resistance to etoposide toxicity. EMT6 cells were treated with either 10 μ g/ml brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h, hypoxia (HYX) for 8 h, or okadaic acid (OA) for 8 h. Etoposide (10, 25, or 50 μ M) was added during the last hour of stress treatment before analysis by colony-forming assay. The toxicity of etoposide in nonstressed cells (CON) is also shown. Results shown are the mean percentage of control cell survival \pm S.E.M. from three to five independent experiments.

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express levels of wild-type I κ B α comparable with that of nontransfected EMT6 cells. However, I κ B α M cells treated with ponasterone A for 24 h express the slower-migrating I κ B α M only. We confirmed these findings by reprobing these blots with an anti-HA primary antibody (Fig. 2). Only ponasterone-induced I κ B α M cells express protein that is immunoreactive to the anti-HA antibody. The absence of wild-type I κ B α in extracts from induced I κ B α M cells may be explained by the rapid association and dissociation of NF- κ B/I κ B α complexes (Schmid et al., 2000). Over the 24 h of ponasterone A treatment, NF- κ B/I κ B α M complexes become prevalent because I κ B α M is not sensitive to I κ B kinases and subsequent proteasome degradation (DiDonato et al., 1996). I κ B α that is not bound to NF- κ B, as a result of increasing competition with I κ B α M, is degraded (Henkel et al., 1993) and therefore is not present at levels sufficient for detection by Western blot analysis.

I κ B α M Prevents Stress-Induced Activation of NF- κ B.

To determine whether the expressed I κ B α M was functionally active, we tested whether I κ B α M expression could inhibit NF- κ B activation. Cells were exposed to BFA, hypoxia, or OA stress, and nuclear extracts were prepared at times shown previously to correspond with maximal stress-induced NF- κ B activation (hypoxia for 2 h, 60 nM okadaic acid for 8 h, or 10 μ g/ml BFA for 2 h, followed by 2 h in BFA-free media) (Lin et al., 1998) and assayed for the presence of free NF- κ B by EMSA. Our results show that BFA, OA, and hypoxia all induce NF- κ B activation in both VCT cells treated with ponasterone A and noninduced I κ B α M cells (Figs. 3A and 4A). In our results, two bands of specific binding are detectable, which others have suggested are the p65/p50 (upper band) and p50/p50 forms (lower band) of NF- κ B (Conant et al., 1994). I κ B α M cells pretreated with ponasterone A, however, had greatly reduced levels of BFA-, OA-, and hypoxia-induced NF- κ B activation (Figs. 3A and 4A). To demonstrate the specificity of DNA binding, we performed competition experiments with nonlabeled NF- κ B or AP-1 oligonucleotides. Figures 3B and 4B show that the addition of a 50-fold excess of NF- κ B oligonucleotide effectively blocks the specific

interactions of NF- κ B with the radiolabeled probe. The AP-1 oligonucleotide is the same length as the NF- κ B oligonucleotide, but it is otherwise not related in sequence identity. The addition of a 50-fold excess of AP-1 oligonucleotide resulted in no change in binding of NF- κ B to the labeled probe (Figs. 3B and 4B). Taken together, these results show that BFA, OA, and hypoxia activate NF- κ B and that I κ B α M expression prevents stress-induced formation of free NF- κ B in the nucleus.

To test whether I κ B α M expression inhibits NF- κ B function, we transiently transfected cells with an NF- κ B-sensitive luciferase reporter plasmid, pTk-(κ B)₆-Luc. This plasmid contains a luciferase reporter gene downstream of a thymidine kinase promoter with six κ B binding sites for NF- κ B (Bachelerie et al., 1991). After transfection, cells were treated with ponasterone A to induce I κ B α M expression and then were stress-treated (in the presence of ponasterone A) with

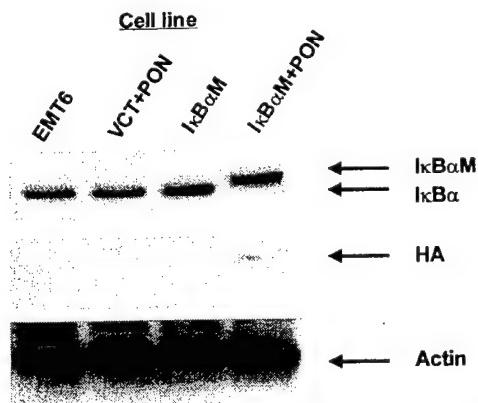


Fig. 2. Western blot analysis of inducible I κ B α M expression. EMT6 cells (EMT6) were transfected with either pIND and pVgRXR (VCT) or pVgRXR and pIND-I κ B α M (I κ B α M). The I κ B α M also contains three HA tags, which increase the molecular weight of I κ B α M relative to wild-type I κ B α . After treatment with 10 μ M ponasterone A (PON) for 24 h, whole-cell lysates were collected and analyzed by Western blot. The resulting membranes were probed with anti-I κ B α (top), anti-HA (middle), and anti-actin (bottom) primary antibodies. Shown is a representative blot from one of three independent experiments.

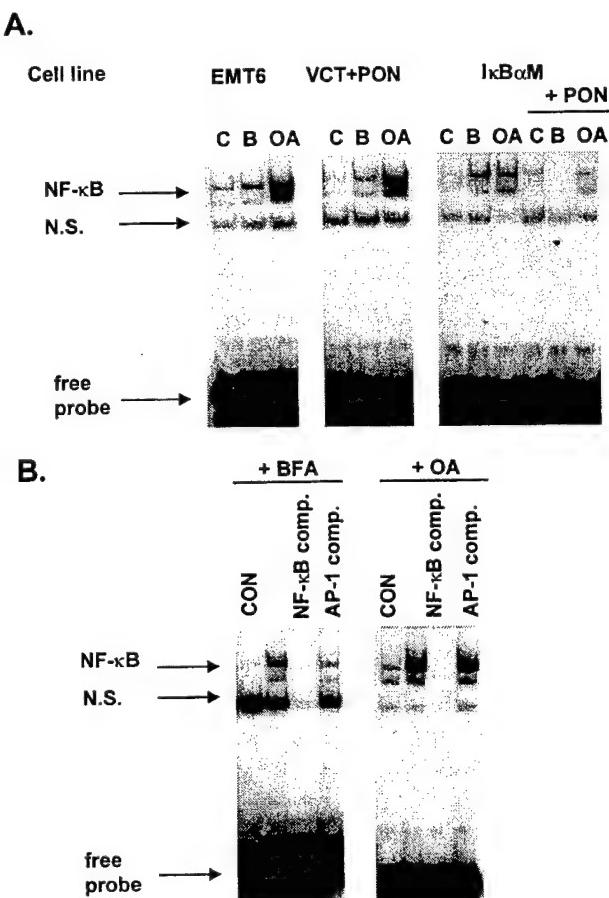


Fig. 3. A, I κ B α M expression prevents chemical stress-induced activation of NF- κ B. VCT and I κ B α M cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h. VCT, I κ B α M, and nontransfected EMT6 cells (EMT6) were stress-treated in the presence of PON with 10 μ g/ml brefeldin A (B or BFA) for 2 h, followed by a BFA-free recovery for 2 h or 60 nM okadaic acid (OA) for 8 h (stress treatments previously shown to cause maximal NF- κ B activation). After drug treatments, nuclear extracts from stress-treated and nonstressed (C) cells were harvested and analyzed by EMSA using a 32 P-labeled NF- κ B oligonucleotide. Specific binding of NF- κ B to the probe (NF- κ B), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of NF- κ B binding by competition assay. Nuclear extracts from nonstressed (CON), BFA-, and OA-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF- κ B or AP-1 oligonucleotide (NF- κ B comp. and AP-1 comp., respectively).

either hypoxia for 2 h, 60 nM OA for 8 h, or 10 μ g/ml BFA for 2 h, followed by a 2 h recovery in BFA-free media (time points shown previously to correspond with maximal stress-induced NF- κ B activation). Cells were lysed, collected, and analyzed for luciferase expression by determining the light emission per sample in the presence of luciferol substrate. The relative luciferase activity obtained is indicative of the relative amount of functional NF- κ B for a given drug treatment. Figure 5 shows that treatment of EMT6 cells with BFA, hypoxia, or OA results in a marked increase in luciferase activity compared with activity observed in nonstressed cells (Fig. 5, A and B). VCT and I κ B α M cells had similar increases in luciferase activity with stress treatment (Fig. 5, A and B). In contrast, I κ B α M cells treated with ponasterone A had significantly less BFA-, hypoxia-, and OA-induced luciferase activity (Fig. 5, A and B). These results suggest that I κ B α M expression blocks the formation of stress-induced free nuclear NF- κ B

and prevents enhanced NF- κ B trans-activation caused by stress treatment.

I κ B α M Expression Does Not Alter Etoposide Cytotoxicity in the Absence of Stress. Recent studies have reported that inhibition of NF- κ B activation enhances the toxicity of anticancer agents (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). To determine the effects of I κ B α M expression on etoposide cytotoxicity in the absence of stress, VCT and I κ B α M cells were induced with ponasterone A for 24 h and then treated with etoposide for 1 h before analysis by colony-forming assay. Figure 6 shows that VCT cells treated with ponasterone A, noninduced I κ B α M cells, and I κ B α M cells treated with ponasterone A did not have significant changes in cell survival in the

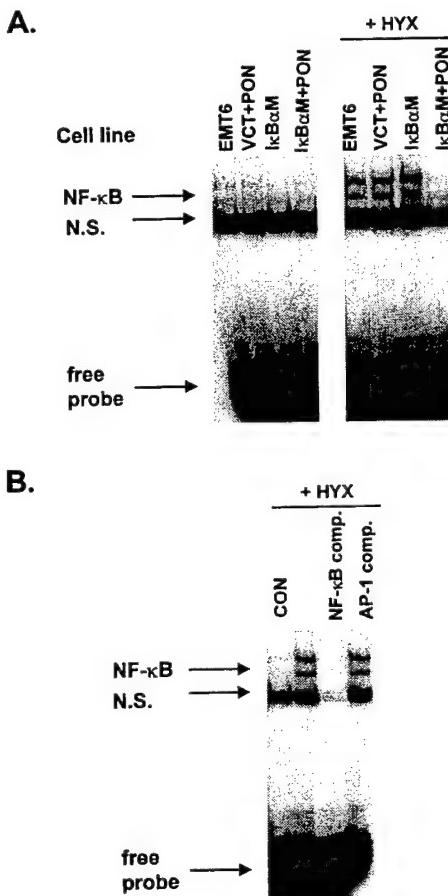


Fig. 4. A, I κ B α M expression prevents hypoxia-induced activation of NF- κ B. VCT and I κ B α M cells were treated with 10 μ M ponasterone A (PON) for 24 h. VCT, I κ B α M, and nontransfected EMT6 cells (EMT6) were then stress-treated with hypoxia (HYX) for 2 h (a stress treatment previously shown to cause maximal NF- κ B activation). After drug treatments, nuclear extracts were harvested and analyzed by EMSA using a 32 P-labeled NF- κ B oligonucleotide. Specific binding of NF- κ B to the probe (NF- κ B), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of NF- κ B binding by competition assay. Nuclear extracts from nonstressed (CON) and hypoxia-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF- κ B or AP-1 oligonucleotide (NF- κ B comp. and AP-1 comp., respectively).

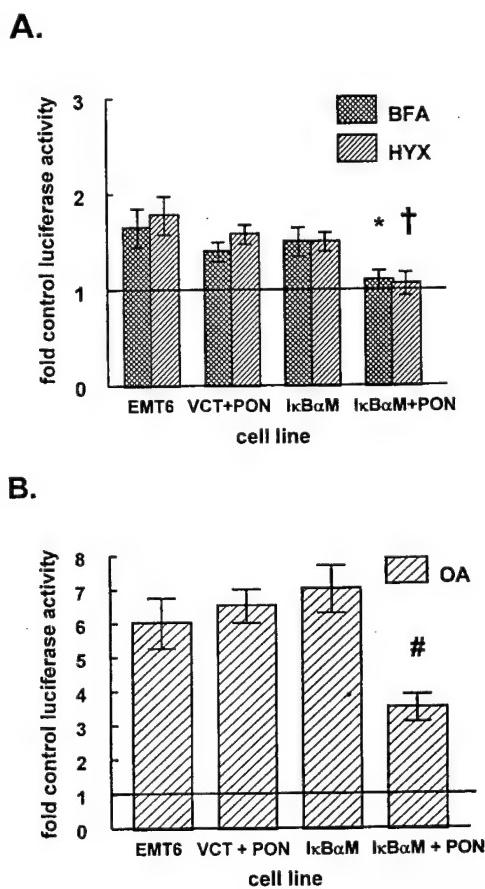


Fig. 5. I κ B α M expression inhibits stress-induced NF- κ B transactivation. EMT6, VCT, and I κ B α M cells were transiently transfected with a NF- κ B-sensitive luciferase reporter gene. After transfection, cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h to induce gene expression. Cells were stress-treated in the presence of M ponasterone A with either 10 μ g/ml brefeldin A (BFA) for 2 h followed by a recovery in BFA-free media for 2 h, hypoxia (HYX) for 2 h (A), or 60 nM okadaic acid (OA) for 8 h (B) (time points previously shown to cause maximal NF- κ B activation). Results shown are the average fold-control luciferase activities from three to five independent experiments. Bars, S.E.M. *, a statistically significant decrease in luciferase activity was observed in BFA-treated I κ B α M cells induced with PON compared with noninduced BFA-treated I κ B α M cells. †, a statistically significant decrease in luciferase activity was observed in hypoxia-treated I κ B α M cells induced with PON compared with noninduced HYX-treated I κ B α M cells. #, a statistically significant decrease in luciferase activity was observed in OA-treated I κ B α M cells induced with PON compared with noninduced OA-treated I κ B α M cells ($p < 0.05$, ANOVA).

presence of etoposide compared with nontransfected EMT6 cells.

I κ B α M Expression Prevents BFA-, Hypoxia-, and OA-Induced Resistance to Etoposide. Our preliminary data with the proteosome inhibitor MG-132 suggested that inhibition of NF- κ B activation could reverse stress-induced resistance (Lin et al., 1998). To determine whether NF- κ B activation mediates stress-induced drug resistance, we assessed whether I κ B α M expression could prevent BFA-, hypoxia-, and OA-induced resistance to etoposide. I κ B α M cells were treated first with ponasterone A for 18 h and then with a stress treatment of hypoxia for 8 h, 60 nM OA for 8 h, or 10 μ g/ml BFA for 2 h, followed by a recovery for 6 h in BFA-free media (in the continued presence of ponasterone A). During the last hour of stress, cells were treated with etoposide before analysis by colony-forming assay. Figure 7 shows that noninduced I κ B α M cells treated with BFA (Fig. 7A), hypoxia (Fig. 7B), or OA (Fig. 7C) are resistant to the cytotoxic effects of etoposide. Etoposide cytotoxicity in induced VCT cells treated with stress was not statistically different from that observed in noninduced I κ B α M cells treated with stress (data not shown). However, ponasterone-induced I κ B α M cells treated with BFA, hypoxia, or OA (Fig. 7, □) were significantly more sensitive to the cytotoxic effects of etoposide compared with noninduced I κ B α M cells (Fig. 7, ■). I κ B α M expression partially but significantly reversed BFA-induced resistance to etoposide, whereas the reversal of hypoxia- and OA-induced drug resistance was almost complete. At nearly all doses of etoposide, the cell survival of induced I κ B α M cells treated with hypoxia or OA was not significantly different from the cell survival of nonstressed I κ B α M cells treated with etoposide alone. These data indicate that specific inhibition of NF- κ B attenuates both chemical- and physiological-induced resistance to etoposide.

Discussion

Most solid tumors are resistant to chemotherapy. This drug resistance has been attributed, in part, to the unique physiology of solid tumors. Oxygen deficiency (hypoxia), glucose deprivation, and acidosis are widespread conditions in

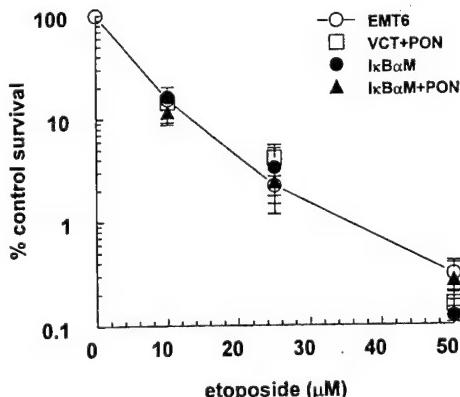


Fig. 6. I κ B α M expression and stable transfection do not alter etoposide cytotoxicity in the absence of stress. VCT, I κ B α M, and nontransfected EMT6 cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h. Etoposide (10, 25, or 50 μ M) was added during the final hour of PON treatment before analysis by colony-forming assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. bars, S.E.M.

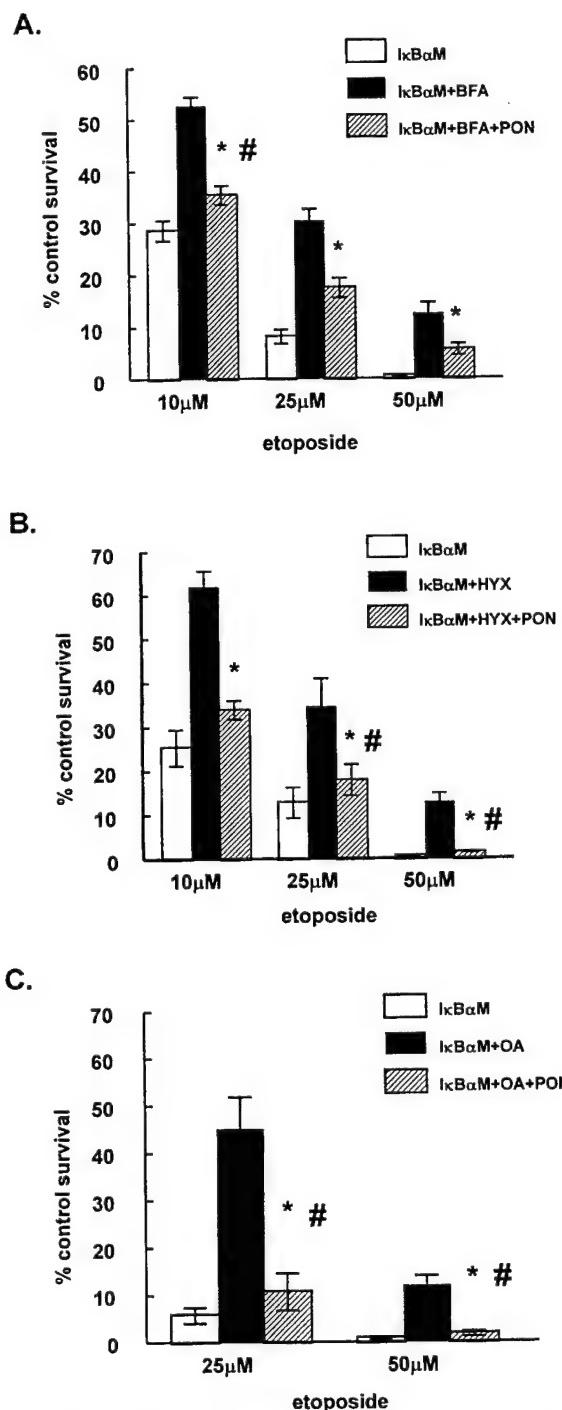


Fig. 7. I κ B α M expression prevents BFA-, HYX-, and OA-induced resistance to etoposide. I κ B α M cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h. Cells were stress-treated in the presence of PON with 10 μ g/ml brefeldin A (BFA) for 2 h, followed by a recovery in BFA-free media for 6 h (A), hypoxia for 8 h (B), or 60 nM okadaic acid (OA) for 8 h (C). Etoposide (10, 25, or 50 μ M) was added during the final hour of PON and stress treatment before analysis by clonogenicity assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. Bars, S.E.M. *, a statistically significant decrease in survival of induced I κ B α M cells treated with stress was observed compared with noninduced I κ B α M cells treated with stress. #, there was no statistically significant change in survival of induced I κ B α M cells treated with stress compared with nonstressed I κ B α M cells ($p < 0.05$, ANOVA).

solid tumors. Hypoxia has been shown repeatedly to limit the responsiveness of tumor cells to ionizing radiation (Bush et al., 1978) and chemotherapeutic agents (Sakata et al., 1991). Mechanisms by which hypoxic cells develop resistance to radiation and chemotherapy may involve low oxygen tension and poor drug penetration into solid tumors (Durand, 1989). Hypoxia treatment is also known to cause gene amplification, cell-cycle arrest, and altered cell-cycle distribution (Stoler et al., 1992; Amellem and Pettersen, 1997). Alterations in gene expression and cell-cycle progression mediated by hypoxia-activated proteins, such as the hypoxia-inducible factor, HIF-1 α (Carmeliet et al., 1998), may be involved in the resistance of tumor cells to cancer chemotherapeutic drugs.

Another type of resistance that develops in cells exposed to hypoxia may be associated with the induction of specific stress-responsive proteins and transcription factors. At the cellular level, the ER responds to stress by three distinct signaling mechanisms. One pathway, the unfolded protein response (UPR), is activated by the presence of abnormally folded proteins in the ER and results in production of the glucose-regulated protein GRP78 (Pahl, 1999). The EOR results in activation of the nuclear transcription factor NF- κ B by the accumulation of normally folded proteins in the ER (Pahl, 1999). The third, the sterol regulatory cascade, is induced by the depletion of cholesterol (Pahl, 1999).

Evidence now suggests that the activation of ER stress pathways may explain the intrinsic insensitivity of solid tumors to chemotherapy. Stress conditions associated with solid tumors, such as hypoxia, induce the expression of glucose-regulated proteins (Wilson et al., 1989), heat-shock proteins (Patel et al., 1995), stress-activated protein kinases (Conrad et al., 2000), and NF- κ B (Koong et al., 1998) and resistance to anticancer agents (Wilson et al., 1989). Analysis of human breast tumors has determined that GRP78 levels are elevated in malignant but not in nonmalignant lesions (Fernandez et al., 2000). The activation of ER stress responses has been further correlated with the development of resistance to anticancer agents that inhibit topoisomerase II. Treatment with the glucose-regulated stresses 2-deoxyglucose, glucosamine, calcium ionophore, or tunicamycin results in activation of NF- κ B (Pahl and Baeuerle, 1997; Pahl, 1999) and the development of resistance to teniposide, etoposide, and doxorubicin (Adriamycin) (Hughes et al., 1989; Lin et al., 1998). In the present study, we show that EMT6 cells treated with other chemical stress agents, BFA or OA, or the physiological stress agent hypoxia, result in similar levels of resistance to etoposide. This finding implies that the mechanism of stress-induced resistance to etoposide may be through the activation of ER stress responses.

Our previous work suggests that the EOR pathway mediates stress-induced resistance to etoposide. We have shown that BFA treatment induces both the UPR and EOR stress pathways and causes the development of resistance to the topoisomerase II inhibitor teniposide (Lin et al., 1998). This study showed that selective activation of the EOR pathway with OA also results in the development of resistance to teniposide to an extent similar to that observed with BFA treatment (Lin et al., 1998). Selective activation of the UPR pathway with the glucosidase inhibitor castanospermine resulted in no change in sensitivity to teniposide even though it markedly increased GRP78 levels (Lin et al., 1998). Furthermore, inhibition of NF- κ B activation with MG-132 or prosta-

glandin A₁ is sufficient to reverse BFA-induced resistance to teniposide (Lin et al., 1998; manuscript in preparation). Taken together, these data suggested the hypothesis that activation of the EOR pathway through the release of NF- κ B is the mechanism by which EMT6 cells develop resistance to etoposide.

To study the role of the EOR pathway in stress-induced drug resistance, a phosphorylation site-deficient mutant of I κ B α (I κ B α M) was used to selectively inhibit NF- κ B activation. Inducible expression of I κ B α M resulted in virtually no detectable wild-type I κ B α . When introduced into cells, I κ B α M probably becomes the major NF- κ B/I κ B α complex because of the high on-off rate of NF- κ B/I κ B α binding kinetics (Schmid et al., 2000). Over time, NF- κ B/I κ B α M complexes become predominant over NF- κ B/I κ B α complexes because I κ B α M cannot be phosphorylated by I κ B kinases and degraded (DiDonato et al., 1996). Free I κ B α that has dissociated from NF- κ B is degraded by proteasomes and thus does not appear in protein collections from I κ B α M cells treated with ponasterone A for 24 h (Henkel et al., 1996).

In this study, we show that expression of I κ B α M suppresses stress-induced NF- κ B activation. Previous time-course studies in our laboratory have shown that maximal stress-induced NF- κ B activation occurs 2 h after hypoxia treatment, 4 h after BFA treatment, and 8 h after OA treatment (Lin et al., 1998; data not shown). These time points were used in the present study to ascertain whether I κ B α M expression could inhibit the maximal NF- κ B response to stress. In both EMSA and luciferase reporter gene assays, VCT cells treated with ponasterone A and noninduced I κ B α M cells display enhanced NF- κ B activation with stress. Ponasterone A treatment alone does not activate NF- κ B or interfere with stress-induced NF- κ B activation in EMT6 cells (data not shown). Despite the differences in NF- κ B activation kinetics, I κ B α M cells treated with ponasterone A were virtually insensitive to stress-induced NF- κ B activation. The expression of I κ B α M also greatly inhibited NF- κ B activation induced by OA, a relatively stronger activator of NF- κ B (Lin et al., 1998). These data show that the inducible I κ B α M was useful for testing the effects of selective inhibition of NF- κ B on drug resistance.

The effects of I κ B α M expression on etoposide toxicity were determined. Others have observed that I κ B α M expression enhances the toxicity of anticancer agents such as camptothecin, paclitaxel, daunorubicin, and tumor necrosis factor (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). However, we observed no significant change in cell survival of induced I κ B α M cells treated with etoposide compared with noninduced I κ B α M cells. These data suggest that NF- κ B activation does not influence the cytotoxicity of topoisomerase II inhibitors in the absence of stress in our murine cell line.

We have demonstrated that inducible I κ B α M expression prevents drug resistance caused by BFA, hypoxia, and OA. Noninduced I κ B α M cells exhibit levels of BFA-, hypoxia-, and OA-induced resistance to etoposide similar to those levels observed in wild-type EMT6 cells. Induction of I κ B α M, in contrast, results in significantly greater drug toxicity in the presence of stress. The abrogation of drug resistance was essentially complete, because hypoxia- or OA-treated I κ B α M cells induced with ponasterone A had etoposide toxicity levels

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that were not significantly different from those of non-stressed cells.

Taken together, our data clearly show that NF- κ B activation plays a critical role in both chemical and physiological resistance to etoposide. Although statistically significant, the reversal of stress-induced drug resistance with I κ B α M expression was not complete with the stress agent BFA. However, reversal of hypoxia- or OA-induced resistance was complete. Although there is evidence that I κ B kinases, which play a primary role in I κ B phosphorylation, may activate additional signaling pathways (Hu et al., 2001), evidence for the direct effects of I κ B α on other signaling pathways is not available. Other pathways independent of NF- κ B activation may contribute to stress-induced drug resistance, but the data presented here show that NF- κ B activation plays a major role in stress-induced drug resistance. Our data further imply that relatively small changes in NF- κ B activation can have dramatic effects on cell viability, suggesting that inhibition of NF- κ B activation may result in the modulation of pleiotropic responses with biological and therapeutic significance. The concentrations of etoposide used in our studies are within the range of plasma concentrations of etoposide that are obtained clinically (Chen and Uckun, 2000). Therefore, it is likely that stress-induced resistance to topoisomerase II inhibitors play a role in the intrinsic resistance of solid tumors to topoisomerase II-directed agents. These findings also suggest that coadministration of agents that inhibit the activation of NF- κ B would enhance the efficacy of topoisomerase II inhibitors in the treatment of cancer. Inhibition of NF- κ B activation with I κ B α M is known to enhance the toxicity of many anticancer agents both in vivo and in vitro (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). Agents that inhibit NF- κ B activation, such as the proteasome inhibitors PS-341 and lactacystin, enhance chemotherapeutic efficacy in in vivo tumor assays (Teicher et al., 1999; Ogiso et al., 2000). These studies and those reported here suggest that the interruption of signaling pathways mediating intrinsic drug resistance, such as physiological stress, represents new therapeutic targets for cancer drug therapy.

Acknowledgments

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Prostaglandin A₁ Inhibits Stress-Induced NF-κB Activation and Reverses Resistance to Topoisomerase II Inhibitors

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Abstract. Stress conditions associated with solid tumors lead to the formation of heterogeneous tumor cell subpopulations and insensitivity to cancer chemotherapeutics. In this report, we show that EMT6 mouse mammary tumor cells treated with the chemical stress, brefeldin A (BFA), or the physiological stress, hypoxia, develop resistance to the topoisomerase II (topoII) inhibitors teniposide and etoposide. BFA and hypoxia treatment did not alter intracellular drug concentrations, topoII protein levels, or inhibit topoII activity. BFA and hypoxia did cause the activation of the nuclear transcription factor NF- κ B. We demonstrate that pretreatment with the synthetic cyclopentenone prostaglandin A₁ (PGA₁) inhibits stress-induced NF- κ B activation and reverses BFA- and hypoxia-induced resistance. The reversal of BFA-induced resistance can occur when PGA₁ is administered either before or several hours after the induction of stress. Taken together, these data support the involvement of NF- κ B in stress-induced drug resistance, show that pharmacologic inhibitors of NF- κ B can disrupt the biological consequences of stress and imply that inhibitors of NF- κ B may be useful agents to enhance the clinical efficacy of topoII-directed chemotherapeutics.

FOOTNOTES

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^eAbbreviations used: BFA, brefeldin A; HYX, hypoxia; topoII, topoisomerase II; NF- κ B, nuclear factor- κ B; PGA₁, prostaglandin A₁; ER, endoplasmic reticulum; GRP78, glucose regulated protein 78; I κ B, inhibitory NF- κ B protein; EOR, ER overload response; MG-132, carbobenzyoyl-leucinyl-leucinyl-leucinal; OA, okadaic acid; kDNA, kinetoplast DNA; EMSA, electrophoretic mobility shift assay; TK, thymidine kinase.

INTRODUCTION

The development of drug resistance is a major obstacle to the successful treatment of human cancers. Resistance of tumors to chemotherapeutic agents which inhibit the topoisomerase II (topoII)^e enzyme is often an acquired phenotype that develops in response to repeated drug administration (1). The most common mechanism of acquired resistance is through expression of the 170 kDa P-glycoprotein or 190 kDa transmembrane transport protein which prevent intracellular drug accumulation (2, 3). Resistance to topoII inhibitors has also been correlated with decreased levels of topoII or decreased topoII activity (4).

A nonacquired form of resistance to topoII-directed agents also has been identified. Solid tumors often have inadequate vascularization due to the progressive expansion of malignant cells. Poor blood flow causes the formation of hypoxic, acidic and/or nutrient-deprived cell subpopulations that can have altered growth and functional properties (5, 6). Stress conditions such as glucose deprivation and hypoxia are known to cause intrinsic tumor cell resistance to topoII inhibitors (7-9). We and others have demonstrated that intrinsic resistance to topoII inhibitors can also be induced by the activation of stress-responsive proteins through the inhibition of protein glycosylation, release of endoplasmic reticulum (ER) calcium stores, or disruption of ER-to-Golgi transport (10, 11). These findings suggest that the pathways activated by ER stress responses may mediate physiologically-induced resistance to topoII inhibitors.

Resistance to topoII inhibitors has been associated with the induction of the 78 kDa glucose-regulated stress protein (GRP78). Treatment with 2-deoxyglucose, glucosamine, tunicamycin, brefeldin A (BFA), calcium ionophores, glucose deprivation, or hypoxia has been correlated with the induction of GRP78 and the development of intrinsic resistance to topoII inhibitors (10-13). However, evidence now suggests that GRP78 induction is not sufficient to

cause drug resistance. We have shown EMT6 mouse mammary tumor cells treated with the glucosidase inhibitor castanospermine exhibited elevated GRP78 levels, but remained sensitive to teniposide (11). Inhibition of GRP78 with an antisense oligodeoxynucleotide resulted in no alteration in stress-induced apoptosis by the glycosylation inhibitor tunicamycin (13).

Another ER stress response pathway has been characterized that involves the nuclear transcription factor NF- κ B. NF- κ B is a heterodimeric transcriptional activator protein often bound to a cytosolic inhibitory I κ B protein (14-16). To activate NF- κ B, I κ B must be phosphorylated, ubiquitinated and then degraded by proteasomes (15, 16). After degradation of I κ B, NF- κ B translocates into the nucleus where it binds to consensus elements and directs the transcription of NF- κ B-dependent genes (17). Our laboratory and others have shown that a variety of physiological and chemical stress conditions result in the activation of NF- κ B (11, 18). Accumulation of protein in the ER by stress causes the release of intracellular Ca²⁺ from the ER, the formation of reactive oxygen species and the activation of NF- κ B by the ER-overload pathway (EOR) (19).

NF- κ B activation is also known to protect cells from apoptosis and the toxic effects of anticancer agents. NF- κ B activation inhibits caspase 8 activation through the regulation of tumor necrosis factor receptor-associated factor (TRAF) and inhibitor of apoptosis (IAP) proteins (20) and prevents cytochrome *c* release through activation of the A1/Bfl-1 gene product, a Bcl-2 family member (21). Inhibitors of NF- κ B activation are known to enhance the toxicity of tumor necrosis factor, paclitaxel, daunorubicin, and camptothecin on tumor cells (22-24). These findings suggest that stress-induced NF- κ B activation may cause resistance to topoII inhibitors

by preventing apoptosis and that inhibition of NF- κ B activation may reverse stress-induced drug resistance.

We have shown that treatment with the chemical stress agent BFA causes NF- κ B activation and the development of resistance to the topoII inhibitor teniposide (11). BFA induces stress through the EOR pathway by disrupting protein transport from the ER to the Golgi apparatus (25). Pretreatment with the proteasome inhibitor MG-132 blocks BFA-induced activation of NF- κ B and reverses BFA-induced resistance to teniposide (11). To extend these findings, we investigated the effects of another pharmacologic inhibitor of NF- κ B activation on stress-induced drug resistance. Prostaglandin A₁ (PGA₁) is a synthetic cyclopentenone prostaglandin that has been used therapeutically to inhibit DNA and RNA viruses and prevent the growth of a variety of tumors (26, 27). PGA₁ is also a potent inhibitor of NF- κ B activation by preventing phosphorylation of I κ B and causing upregulation of I κ B (28, 29).

In this study, we demonstrate treatment with the chemical stress, BFA or the physiologic stress, hypoxia results in NF- κ B activation and resistance of EMT6 mouse mammary tumor cells to teniposide and etoposide. BFA or hypoxia treatment alone did not alter intracellular etoposide accumulation, topoII levels, or topoII activity, suggesting stress-induced resistance is not mediated by the common mechanisms of acquired drug resistance. To investigate the role of NF- κ B in stress-induced resistance, we have tested if a pharmacologic inhibitor of NF- κ B could reverse BFA- and hypoxia-induced resistance. Pretreatment with PGA₁ inhibits stress-induced activation of NF- κ B and reverses BFA- and hypoxia-induced resistance to teniposide and etoposide. Furthermore, we demonstrate that BFA-induced resistance can be reversed with PGA₁ even when the reversal agent is given several hours after the induction of stress.

MATERIALS AND METHODS

Cell culture. EMT6 mouse mammary tumor cells (provided by Dr. S. Rockwell, Yale University, New Haven, CT) were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (Invitrogen, San Diego, CA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml gentamicin sulfate (Biofluids, Rockville, MD). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/ 95% air and passaged every 3-4 days.

Reagents and Treatments. To induce stress, cells were treated with 10 µg/ml brefeldin A (BFA) (Sigma) for 2 h followed by an incubation in BFA-free media (luciferase assay and EMSA) or for 2 h followed by an incubation in BFA-free media for 6 h (all other experiments). Cells were also treated with 5 µg/ml tunicamycin (Sigma) for 6 h, 10 mM 2-deoxy-D-glucose (Sigma) for 18 h, 10 mM glucosamine (Sigma) for 18 h, or 60 nM okadaic acid (OA) (Calbiochem, San Diego, CA). For hypoxia, cells were seeded in gas-impermeable glass flasks and then exposed to continuous hypoxia as described previously (30) for either 2 h (luciferase assay and EMSA), 8 h (colony forming experiments), or 18 h (drug uptake and western blot). To reverse drug resistance, 25 µM prostaglandin A₁ (PGA₁) (Biomol, Plymouth Meeting, PA) was added one-half hour prior to the induction of stress, concurrent with the addition of BFA, or 30 min, 1 h, 2 h, 4 h, 6 h, or 7 h after the induction of BFA stress. Teniposide (Bristol Myers, Syracuse, NY) and etoposide (Sigma) were added during the final hour of stress treatment prior to analysis by colony forming assay.

Drug Uptake Measurements. Cellular uptake of radiolabeled etoposide was measured as previously described (31). During the final hour of stress treatment cells were incubated in 50

μ M unlabeled etoposide, 2.5 μ Ci (2.78 nM) of [3 H]-labeled etoposide (900 mCi/ mmol, Moravek Biochem, Brea, CA) and 0.5 μ Ci of [14 C]-labeled inulin. To ensure complete removal of extracellular [3 H]-labeled etoposide, radiolabeled inulin levels were monitored. Unlabeled etoposide (50 μ M) was added to the washes to prevent loss of intracellular etoposide stores. After washing, cells were lysed in 1N NaOH, neutralized with 1N HCl, plated in Ecolite scintillation cocktail (ICN, Costa Mesa, CA) and counted by scintillation spectrometry. For each experiment, cell counts were performed on triplicate tissue culture plates for each stress treatment and radiolabeled etoposide uptake was expressed as picomoles per 10^6 cells.

Whole Cell and Nuclear Extraction of TopoII. Following stress treatment, whole cell lysates were collected by suspending cell pellets in 90 μ l of 1X DNase/RNase (with 10 μ g/ml each of antipain, aprotinin, chymotrypsin, leupeptin, and pepstatin A and 35 μ g/ml PMSF) and 10 μ l 10% SDS. After incubation on ice for 60 min, 60 μ l of SDS (8% SDS, 200 mM Tris-Cl, 40% glycerol, 400 mM DTT) was added and the samples were boiled for 5 min. For nuclear extraction, cells were washed in Buffer A (0.15 M NaCl, 10 mM KH₂PO₄, pH 7.5) and incubated for 20 min in Buffer B (10 mM Tris, 1.5 mM MgCl₂, 10 mM NaCl, 4 mM DTT, and 1 mM PMSF, pH 7.5) at 4°C. Next, 1 ml of Nonidet detergent was added and the cells were gently triturated and incubated at 4°C for 15 min. Cells were lysed using dounce homogenization for 20 strokes and then centrifuged at 2500g for 10 min. The pellet was resuspended in 2 ml of Buffer C (50 mM Tris, 25 mM KCl, 2 mM CaCl₂, 3 mM MgCl₂, 0.25 M sucrose, 4 mM DTT, 1 mM PMSF, pH 7.5) and then layered over Buffer D (Buffer C with 0.6 M sucrose). Cell nuclei were isolated by centrifugation at 6000g for 10 min at 4°C. The nuclear pellet was resuspended in 1 ml of Buffer E (50 mM Tris, 25 mM KCl, 3 mM MgCl₂, 4 mM DTT, 1 mM PMSF, pH 7.5) and

incubated for 15 min at 4°C. An equal volume of Buffer F (50 mM Tris, 2 M NaCl, 4 mM DTT, 10 mM disodium EDTA, 1 mM PMSF, pH 7.5) was added and the samples were incubated for 30 min at 4°C. Finally, 10% glycerol was added and the protein extracts were collected after ultracentrifugation at 100,000g for 60 min.

Western Blot Analysis. Nuclear extract (50-100 µg) or whole cell lysate (2 mg total protein) was mixed with 2X SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, 2% β-mercaptoethanol) and boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel (4% stacking gel, pH 6.8, 10% resolving gel, pH 8.8, 30:0.8 acrylamide:bisacrylamide) and transferred to a nitrocellulose membrane. After blocking in 1X TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) with 5% non-fat dry milk, the membrane was incubated with a 1:1000 dilution of primary polyclonal rabbit antibody against p170 topoIIα (provided by Dr. D. Sullivan, Univ. of S. Florida, Tampa, FL) at 4°C. The blot was washed with 1X TBST and incubated with a secondary goat anti-rabbit HRP-conjugated antibody (Promega, Madison, WI) at a dilution of 1:10000 for 1 h at room temperature. Antibody binding was then visualized using an enhanced chemiluminescent reagent (Amersham, Arlington Heights, IL).

In Vitro Human TopoII Assay. Reaction mixtures of 2 units of purified human topoIIα enzyme, 200 ng kinetoplast DNA (kDNA), 1X assay buffer [0.05 M Tris-Cl (pH 8.0), 0.12 M KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, and 30 µg/ml bovine serum albumin] (TopoGEN, Columbus, OH), and either 25 µM PGA₁, 10 µg/ml BFA, or 100 µM teniposide were combined and incubated at 37°C for 1 h. After termination with 10 µl of stop solution (5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol), decatenation products were visualized by gel

electrophoresis on an agarose gel with ethidium bromide and quantitated using an Eagle Eye transilluminator (Stratagene, La Jolla, CA). Percent decatenation was determined by dividing the relative intensity of decatenation product bands by the total lane intensity.

EMSA. Following drug treatment, nuclear extracts were prepared from 1 X 10⁷ cells as previously described (11). Briefly, cells were incubated in 100 µl of lysis buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40, 0.5 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Cell nuclei were washed in 500 µl nuclear washing buffer (lysis buffer without NP-40 added), resuspended in 100 µl of nuclear resuspension buffer [250 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM DTT, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride] and broken by three freeze-thaw cycles. An oligonucleotide of the NF-κB consensus site (Promega) was labeled with 10 mCi/ml [γ -³²P] ATP (6000 Ci/mmol; Amersham, Arlington Heights, IL/Andotek, Irvine, CA) using T4 polynucleotide kinase (Promega) and cleared of unincorporated nucleotides by chromatography through a G-25 spin column (Worthington, Plymouth Meeting, PA). Nuclear extracts (10 µg) were incubated with 4 µg poly dI-dC (Sigma) and 0.035 pmol (50,000-200,000 cpm) of probe in a buffer of 10 mM Tris-HCl (pH 7.5), 50 mM sodium chloride, 0.5 mM EDTA, 1 mM magnesium chloride, 0.5 mM DTT, and 4% glycerol at room temperature for 20 min. Binding was analyzed by autoradiography after electrophoresis on a nondenaturing 6% polyacrylamide gel.

Transient Transfection and Luciferase Reporter Gene Assay. Transfection media containing 3 µg of pTK-6κB-Luc plasmid (provided by Dr. Heike Paul, Univ. Hospital Freiburg, Germany) (32), 1 µg of pcDNA3.1-lacZ (Invitrogen), 12 µl of transFast lipid (Promega), and 2.5

ml of serum-free Waymouth's medium was incubated with cells for 2 h. Following transfection, 2.5 ml of Waymouth's medium containing 30% serum was added. After drug treatments, cells were lysed in 400 μ l Reporter Lysis Buffer (Promega) and 20 μ l of whole cell lysate was combined with 100 μ L Luciferase Assay Reagent containing luciferol (Promega). Light emission in the presence of substrate was measured using a scintillation counter (Beckman). Cell extracts were also assayed for *lacZ* expression by incubating 150 μ l of lysate and 150 μ l of 2X assay buffer containing o-nitrophenyl- β -D-galactopyranoside (Promega) at 37°C for 2 h. The absorbance at 420 nm was measured for each sample and the relative β -galactosidase activity was determined by dividing the activity of drug-treated cell extracts by the activity of solvent-treated cell extracts and then used to correct for variations in transfection efficiency. The fold change in luciferase activity was then calculated by dividing the corrected luciferase activity of drug-treated cells by the corrected luciferase activity of solvent-treated cells.

Colony forming assay. Following drug treatment, cells were serially diluted in Waymouth's medium, seeded in triplicate 60 mm² tissue culture dishes and incubated under maintenance conditions for 7 days as previously described (11). Following incubation, dishes were stained with 0.25% crystal violet and counted. For each treatment, the percent control cell survival was determined by dividing the plating efficiency for a given treatment by the plating efficiency of the appropriate non-treated or solvent-treated control.

Statistics. Statistically significant changes in these data were determined using one way analysis of variance (ANOVA) with multiple comparisons performed using Bonferroni's test with p < 0.05 (33).

RESULTS

Stress conditions associated with tumor microenvironments are known to cause resistance to cancer chemotherapeutics (8-11). To determine whether stress alters the cytotoxicity of topoII inhibitors, we exposed EMT6 cells to the chemical stress agent, BFA, or the physiological stress, hypoxia, prior to treatment with teniposide and etoposide. EMT6 cells were treated with hypoxia for 8 h or with 10 µg/ml BFA for 2 h, followed by an incubation in BFA-free media for 6 h. Teniposide and etoposide were added during the last hour of stress treatment prior to analysis by colony forming assay. Figure 1 shows that cells pretreated with BFA (1A. and 1B.) or hypoxia (1C.) are resistant to the toxicity of teniposide and etoposide.

Our previous data with the proteasome inhibitor MG-132 suggest that NF-κB activation may regulate stress-induced drug resistance (11). To explore the role of NF-κB activation in stress-induced resistance, we treated cells with 25 µM prostaglandin A₁ (PGA₁) 30 min prior to the introduction of stress with BFA and hypoxia. Figure 1 shows that pretreatment with PGA₁ prevents BFA-induced resistance to teniposide and etoposide and hypoxia-induced resistance to teniposide. PGA₁ treatment alone did not alter drug toxicity in the absence of stress (Figure 1).

A common mechanism of resistance to topoII inhibitors is through enhanced drug efflux (2, 3). To test whether stress-induced drug resistance is explained by a decrease in drug concentration, we determined if BFA or hypoxia treatment alter intracellular drug accumulation of radiolabeled etoposide. To allow time for the protein-mediated effects of stress treatment on drug uptake, we treated EMT6 cells with hypoxia for 18 h. Preliminary time course studies in our laboratory have determined that the level of stress-induced resistance to teniposide with 18 h of hypoxia is not statistically different than that observed with 8 h of hypoxia (34). During the

final hour of stress treatment [³H]-labeled etoposide was added. After washing, cell samples were lysed and analyzed for [³H] incorporation by scintillation spectroscopy. Our results show that treatment with BFA (10 µg/ml for 2 h, followed by a BFA-free recovery for 6 h) or hypoxia (18 h) does not result in any significant change in intracellular etoposide concentration (Figure 2).

Normal cellular concentrations of topoII are necessary for the cytotoxicity of topoII inhibitors (4). We have investigated whether stress-induced drug resistance is explained by alterations in total cellular or nuclear levels of the topoII enzyme. EMT6 cells were exposed to various ER stress-inducing agents, including 10 mM 2-deoxy-D-glucose for 18 h, 10 mM glucosamine for 18 h, 10 µg/ml BFA for 2 h followed by a BFA-free incubation for 6 h, 5 µg/ml tunicamycin for 6 h or hypoxia for 18 h. Whole cell and nuclear protein extracts were collected from stress-treated cells and analyzed by western blot using an anti-topoII α p170 polyclonal antibody. Figure 3 shows that stress treatment results in no significant alteration in topoII levels from either whole cell (A.) or nuclear extracts (B.). If anything, increases in whole cell and nuclear topoII were observed. Whole cell extracts from quiescent cells in plateau growth had low levels of topoII (Figure 3A.), as others have observed (35). These data show that stress treatments known to cause resistance to topoII inhibitors do not cause the depletion of total or nuclear topoII in the EMT6 cell line.

To determine whether stressors or drug reversal agents alter topoII activity directly, an *in vitro* topoII-decatenation assay was performed. Purified human topoII α was incubated with coiled kinetoplast DNA (kDNA) in the presence of BFA, PGA₁, or the topoII inhibitor teniposide. The resulting DNA samples were separated by electrophoresis and the percent

decatenation was determined for each treatment. Figure 4 shows that relatively high concentrations of BFA and PGA₁ (10X those used in cell survival assays) did not inhibit topoII-mediated decatenation of kDNA. However, teniposide treatment resulted in a significant inhibition of topoII-mediated kDNA decatenation (Figure 4). This suggests that neither stress nor PGA₁ treatment change the functional activity of topoII. Taken together, Figures 3 and 4 imply that alterations in target enzyme concentration or activity do not explain stress-induced drug resistance or its reversal.

Recent studies have shown that several ER stress-inducing agents activate the transcription factor NF-κB (11, 12). Our previous time course data show that the chemical stress agents BFA or the phosphatase inhibitor okadiac acid (OA) cause marked elevation of NF-κB activation by electrophoretic mobility shift assay (EMSA). These studies suggest maximal stress-induced NF-κB activation occurs after 4 h of BFA treatment or 8 h of OA treatment (11). Here the time-dependence of stress-induced NF-κB activation using the physiological stress hypoxia was determined. Following treatment with hypoxia for 1 to 8 h, nuclear extracts were collected from EMT6 cells, incubated with a radiolabeled NF-κB consensus oligonucleotide and analyzed by gel electrophoresis. Figure 5 shows that hypoxia treatment results in greatly enhanced levels of free nuclear NF-κB, with a maximal response occurring at the 2-hour time point. In our data, two bands of specific binding are detectable which others have suggested are the p65/p50 (upper band) and p50/p50 isoforms of NF-κB (36).

To determine if PGA₁ treatment can inhibit maximal stress-induced NF-κB activation, we analyzed by EMSA nuclear extracts from EMT6 cells treated with 25 μM PGA₁ 30 min prior to treatment with either 10 μg/ml BFA for 2 h followed by 2 h in BFA-free media, hypoxia for 2 h,

or 60 nM OA for 8 h (time points of maximal NF- κ B activation). Figure 6 shows that BFA (A.), hypoxia (B.) or OA (C.) treatment cause greatly enhanced levels of free nuclear NF- κ B. However, PGA₁ pretreatment dramatically inhibited either BFA-, hypoxia- or OA-induced NF- κ B activation (left panel, Figure 6, A.-C., respectively). The specificity of NF- κ B binding was assessed with the addition of a 50-fold excess of non-labeled NF- κ B consensus sequence that effectively blocked specific interaction of BFA-, hypoxia-, or OA-induced free nuclear NF- κ B with the radiolabeled probe (right panel, Figure 6, A.-C., respectively). The addition of a 50-fold excess of AP-1 oligonucleotide resulted in no change in binding of stress-induced NF- κ B to the labeled probe (right panel, Figure 6, A.-C.). Taken together, the data demonstrate that stress induces NF- κ B activation which is blocked by PGA₁ pretreatment.

To determine the effects of stress and PGA₁ on the functional activity of NF- κ B, we transiently transfected EMT6 cells with a NF- κ B-sensitive luciferase reporter construct (pTK-6 κ B-Luc) to measure NF- κ B transactivation. This reporter plasmid contains a luciferase gene downstream of a minimal thymidine kinase (TK) promoter with six NF- κ B binding sites (27). Following transfection, cells were treated with either 10 μ g/ml BFA for 2 h followed by 2 h in BFA-free media or hypoxia for 2 h (time points of maximal NF- κ B activation). Figure 7 shows that cells treated with BFA or hypoxia have enhanced luciferase activity when compared to non-stressed cells. Treatment with 60 nM okadaic acid (OA) for 8 h resulted in an even greater elevation of luciferase activity. To determine the effects of the reversal agent PGA₁ on NF- κ B transactivation, we treated cells with PGA₁ 30 min prior to stress treatment. Our results show that pretreatment with PGA₁ inhibits basal levels of NF- κ B transactivation, as well as BFA-, hypoxia-, and OA-induced NF- κ B activation (Figure 7).

Figure 1 shows that cells treated with BFA are resistant to etoposide and teniposide. This duration of BFA treatment was selected because it results in no alterations in cell survival or DNA, RNA, or protein synthesis (11, data not shown). To determine if shorter BFA exposures result in similar resistance to teniposide, we treated cells with 10 µg/ml BFA for 30 min followed by a BFA-free recovery for 7.5 h. Teniposide was added during the final hour of BFA treatment prior to analysis by colony forming assay. Figure 8 shows that a 30-min exposure to BFA results in a level of resistance to teniposide not significantly different from the standard BFA treatment for 2 h.

PGA₁ can prevent the activation of NF-κB and reverse both BFA- and hypoxia-induced resistance to teniposide (Figures 1, 6, and 7). In these experiments, PGA₁ was added prior to the introduction of stress. *In vivo*, however, tumors are likely exposed to stress before, during, and after chemotherapy is initiated. An agent that could reverse drug resistance after stress has occurred would be more useful therapeutically. We determined whether PGA₁ could prevent drug resistance when used during various phases of the stress response. Cells were exposed to 10 µg/ml BFA for 30 min followed by a BFA-free recovery for 7.5 h. PGA₁ (25 µM) was added either 30 min prior to BFA treatment, at the same time as BFA treatment, or 30 min, 1 h, 2 h, 4 h or 6 h after BFA treatment. Teniposide was added during the last hour of stress and PGA₁ treatment prior to analysis by colony forming assay. In addition, PGA₁ was added 7 h after BFA treatment (and therefore at the same time teniposide was added), 8 h after BFA treatment (1 h after teniposide, immediately before colony forming assay), or 9 h after BFA treatment (teniposide was removed, PGA₁ was added for 1 h before colony forming assay). Figure 9 shows that PGA₁ treatment results in a partial but significant reversal of resistance to teniposide even

when given up to 7 h after BFA treatment. Treatment with PGA₁ 8-9 h after BFA and teniposide treatment failed to inhibit the development of resistance.

DISCUSSION

Solid tumors have a unique physiology that includes the presence of hypoxic and/or glucose-deprived cell subpopulations (6). Hypoxia has been repeatedly shown to limit the responsiveness of tumor cells to ionizing radiation and chemotherapy (7, 8). The mechanism by which hypoxic cells develop resistance to radiation and chemotherapy may involve low oxygen tension and poor drug penetration into solid tumors (37, 38). Genetic and biochemical alterations that develop in hypoxia-treated cells may also contribute to development of drug resistance, especially towards cell cycle-specific chemotherapeutic agents (39, 40). Hypoxia causes changes in genomic stability through the induction of anoxia-responsive endonucleases which result in DNA breakage and altered gene expression (39). Drug insensitivity may also be explained by the influence of hypoxia on cell cycle arrest and distribution through modulation of the p53, p21, and p27 proteins (40).

Recent data suggest the activation of stress responses by hypoxia may also mediate intrinsic tumor drug resistance. Hypoxic conditions induce cellular glucose-regulated stress proteins, heat shock proteins, and stress-activated protein kinases (41-43). Hypoxia also causes the activation of various stress- and redox-sensitive proteins, such as NF- κ B, AP-1, Ref-1 and HIF-1 (18, 44, 45). The direct effects of these proteins on apoptosis and downstream gene expression provides another explanation for how adverse tumor microenvironmental conditions lead to chemotherapeutic drug resistance.

In this report we show that treatment with the chemical ER stress agent, BFA, or the physiologic stress, hypoxia, induce similar levels of resistance to teniposide. These findings imply that the mechanism of hypoxia-induced resistance may be through the induction of ER

stress pathways. Data now suggest there are two major ER-mediated stress response pathways. One pathway, known as the unfolded protein response (UPR), is activated by the presence of abnormally folded proteins in the ER and is characterized by the induction of the glucose-regulated protein GRP78 (19). The second pathway is known as the ER overload pathway (EOR). In this pathway, high levels of protein in the ER cause the release of intracellular calcium stores and activation of the transcription factor NF- κ B (19). Our previous data suggest the EOR pathway mediates stress-induced resistance to topoII inhibitors. We have shown that selective activation of the EOR pathway with okadaic acid is sufficient to cause the development of resistance to teniposide (11). However, selective activation of the UPR pathway with the glucosidase inhibitor castanospermine results in no change in sensitivity to teniposide (11). Hence, the mechanism of stress-induced resistance to topoII inhibitors may be through stimulation of the EOR pathway and NF- κ B activation.

To determine the association between NF- κ B activation and the development of resistance to topoII inhibitors, prostaglandin A₁ (PGA₁) was used. PGA₁ is known to prevent the activation of NF- κ B by inhibiting the phosphorylation and upregulation of the inhibitory I κ B protein (28, 29). Here we demonstrate that PGA₁ treatment effectively blocks NF- κ B activation by BFA, hypoxia or OA treatment. Importantly, PGA₁ prevents BFA- and hypoxia-induced resistance to etoposide and teniposide. These data imply that NF- κ B activation through the EOR pathway may mediate both chemical- and physiologic-induced resistance to topoII inhibitors. The findings presented here further substantiate our previous work with the proteasome inhibitor MG-132 (11). Like PGA₁, MG-132 pretreatment blocks BFA-induced NF- κ B activation and reverses BFA-induced resistance to teniposide (11). The fact that MG-132 and PGA₁ inhibit NF-

κ B activation by different mechanisms and both reverse drug resistance further strengthens the role of NF- κ B activation in stress-induced drug resistance.

In the present study we investigated whether BFA- or hypoxia-induced resistance or its reversal with PGA₁ could be explained by the more common mechanisms of drug resistance. Resistance to topoII inhibitors is often explained by alterations in drug accumulation, topoII levels and/or topoII activity. Our data demonstrate, however, that the accumulation of etoposide was not altered by either chemical or physiologic stress treatment. Similarly, analyses of whole cell and nuclear protein extracts show that topoII protein levels or *in vitro* topoII activity were not altered by chemical or physiologic stress. Others have suggested that PGA₁ treatment inhibits topoII ($IC_{50} = 98 \mu M$) (46), but our data show concentrations of PGA₁ up to 250 μM did not alter topoII-mediated kDNA decatenation. It is therefore unlikely that stress-induced resistance to topoII inhibitors is caused by changes in drug accumulation by the p-glycoprotein or MRP pumps or by altered topoII levels or activity. Moreover, the reversal of stress-induced resistance with PGA₁ cannot be explained by decreased topoII activity.

EMT6 cells exposed to BFA for only 30 min developed resistance to teniposide. This finding suggests that short periods of physiological stress can cause drug resistance. Consequently, even transient losses of blood flow to tumors may have a dramatic impact on whether a tumor will respond to chemotherapeutic treatment. In the present study, cells were exposed to PGA₁ after BFA treatment to determine if drug resistance could be reversed following the induction of stress. PGA₁ pretreatment partially reversed BFA-induced resistance to teniposide when given up to 7 h after stress treatment. These data suggest that PGA₁ is an effective reversal agent not only when given before stress treatment, but even when given several

hours after stress treatment. This implies that the intrinsic drug resistance of solid tumors can be reversed during or after the induction of stress. This finding is especially significant because it is likely that most solid tumors have been or are currently exposed to stress prior to chemotherapy treatment.

It is well known that solid tumors contain stressful microenvironments and are intrinsically resistant and insensitive to cancer chemotherapeutics. Recent data show GRP78 levels are elevated in malignant breast lesions when compared to nonmalignant breast lesions (47). Moreover, the concentrations of etoposide and teniposide we have used lie within or below the ranges of that achieved clinically (48, 49). Therefore, the form of drug resistance characterized here that is induced by physiological and chemical stressors likely exists in solid tumors and may explain in part the intrinsic drug resistance of tumors seen clinically. Our data further suggest that the co-administration of agents which inhibit NF- κ B activation would enhance the efficacy of topoII inhibitors in the treatment of cancer. Others have reported that inhibition of NF- κ B activation enhances the toxicity of anticancer agents both *in vivo* and *in vitro*. Inhibition of NF- κ B activation with proteasome inhibitors increases the toxicity of chemotherapeutics in *in vivo* tumor assays (50, 51). Expression of a mutant I κ B α protein to inhibit NF- κ B activation enhances the sensitivity of tumor cells to apoptotic death by tumor necrosis factor, paclitaxel, and daunorubicin (22, 24). In addition, inducible expression of mutant I κ B α sensitizes physiologic or chemically stress-treated EMT6 cells to etoposide (52). Mutant I κ B α expression *in vivo* reduces growth of head and neck squamous cell carcinoma (53) and sensitizes chemoresistant tumors to the toxic effects of camptothecin (23). The results from experiments with the phosphorylation site-deficient I κ B α mutants demonstrate that NF- κ B

activation plays a prominent role in mediating sensitivity of cells to chemotherapeutic agents (22-24, 52, 53). Taken together, our data suggest that PGA₁ may represent a new class of antitumor agents that improve the clinical efficacy of topoII-directed agents by inhibiting the NF-κB-mediated stress responses that limit the effectiveness of cancer chemotherapeutics.

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FIGURE LEGENDS

Figure 1. PGA₁ reverses BFA- and HYX-induced resistance to topoII inhibitors. EMT6 cells were treated with 25 μ M prostaglandin A₁ (PGA₁) 30 min prior to treatment with 10 μ g/mL brefeldin A (BFA) for 2 h followed by 6 h in BFA-free media (*A.* and *B.*) or hypoxia (HYX) for 8 h (*C.*). Teniposide (*A.* and *C.*) or etoposide (*B.*) were added during the final hour of stress and PGA₁ treatment prior to analysis by colony forming assay. The toxicity of teniposide (*A.* and *C.*) or etoposide (*B.*) treatment in the absence of stress (CON) is also shown. Values represent the mean percent control cell survival calculated from 3-5 independent experiments with three replicates per experiment; *bars*, SEM.

Figure 2. BFA or HYX treatment do not alter cellular etoposide uptake. The uptake of [³H]-labeled etoposide was measured in non-treated (CON) or stress-treated cells. EMT6 cells were treated with either 10 μ g/mL brefeldin A (BFA) for 2 h followed by 6 h in BFA-free media or hypoxia (HYX) for 8 h. During the final hour of stress treatment, [³H]-labeled etoposide (0.5 μ Ci/ml, 5.5 nM) and 0.1 μ Ci [¹⁴C]-labeled inulin in 50 μ M etoposide were added to the cells. Following drug exposure, cells were lysed and analyzed by scintillation spectrometry. Etoposide uptake was determined from the number of counts of radiolabeled etoposide incorporated and converted to picomoles of etoposide per 10^6 cells (pmoles/ 10^6 cells). Values represent the mean drug uptake from three independent experiments with three replicates per experiment; *bars*, SEM.

Figure 3. Stress treatment does not alter topoII protein levels. EMT6 cells were exposed to 10 mM 2-deoxy-D-glucose (**D**) for 18 h, 10 mM glucosamine (**G**) for 18 h, 10 µg/mL brefeldin A (**B**) for 2 h followed by 6 h in BFA-free media, 5 µg/ml tunicamycin (**T**) for 6 h, or hypoxia (**H**) for 18 h. Whole cell (*A*) and nuclear (*B*) protein samples from non-stressed cells (**C**), stress-treated cells, and quiescent cells in plateau growth (**P**) were collected. TopoII protein was detected by western blot analysis using a primary anti-p170 topoII α antibody. Shown is a representative blot from one of three independent experiments (upper panel) and the fold control change in topoII levels quantitated by scanning laser densitometry (lower panel); *bars*, SEM.

Figure 4. BFA and PGA₁ treatment do not alter the function of human topoII *in vitro*. Reaction mixtures of 2 units of purified human topoII α enzyme, 200 ng kDNA, and either 25 or 250 µM prostaglandin A₁ (PGA₁) or 10 or 100 µg/ml brefeldin A (BFA) were incubated at 37°C for 1 h. As a positive control, enzyme and DNA were incubated with 100 µM teniposide (TEN). Decatenation products were separated on an agarose gel with ethidium bromide and quantitated using a Stratagene Eagle Eye transilluminator. Values represent the mean percent control decatenation ± SEM from three independent experiments. * - a statistically significant decrease in decatenation was observed with teniposide treatment when compared to control (one way ANOVA followed by multiple comparison using Bonferroni's test with P < 0.05).

Figure 5. Time course NF-κB activation by HYX stress. EMT6 cells were exposed to hypoxic conditions for 1, 2, 4, 6, or 8 h as described in the materials and methods section. Following treatment, nuclear extracts from non-treated (CON) or hypoxia-treated (HYX) cells

were harvested and analyzed by EMSA using a [³²P]-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), non-specific binding (N.S.) and unbound probe (free probe) bands are indicated.

Figure 6. PGA₁ inhibits stress-induced NF-κB activation. EMT6 cells were treated with 25 μM prostaglandin A₁ (PGA₁) 30 min prior to stress treatment with 10 μg/mL brefeldin A (BFA, A.) for 2 h followed by 2 h in BFA-free media, hypoxia (HYX, B.) for 2 h or 60 nM okadaic acid (OA, C.) for 8 h. Following treatment, nuclear extracts were harvested and analyzed by EMSA using a [³²P]-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), non-specific binding (N.S.) and unbound probe (free probe) bands are indicated. For each experiment, constitutive activation of NF-κB from non-treated cells is also shown (CON). The specificity of NF-κB binding by competition assay was also determined (right panel, A.-C.). Nuclear extracts from BFA-, HYX- or OA-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF-κB or AP-1 oligonucleotide (NF-κB comp and AP-1 comp, respectively). Shown in each panel is one representative gel from three independent experiments.

Figure 7. PGA₁ prevents stress-induced NF-κB transactivation. EMT6 cells were transiently transfected with a NF-κB-sensitive luciferase reporter gene. After transfection, cells were treated with 25 μM prostaglandin A₁ (PGA₁) 30 min prior to stress treatment with 10 μg/mL brefeldin A (BFA) for 2 h followed by 2 h in BFA-free media, hypoxia (HYX) for 2 h or 60 nM okadaic acid (OA) for 8 h (time points of maximal NF-κB activation). Whole cell lysates from non-treated

(CON) and stress-treated cells were assayed for luciferase expression by measuring the fluorescence intensity in the presence of luciferol substrate with scintillation spectroscopy. The fold control luciferase activity was determined by dividing the activity for each treatment by the activity of non-treated cells. Values represent the mean fold control luciferase activity \pm SEM from 3-5 independent experiments. * - a statistically significant decrease in luciferase activity was observed in non-treated and stress-treated cell extracts from cells pretreated with PGA₁ (one way ANOVA followed by multiple comparison using Bonferroni's test with P < 0.05).

Figure 8. Effect of 30-minute BFA exposure on resistance to teniposide. EMT6 cells were exposed to 10 μ g/mL brefeldin A (BFA) for either 30 min followed by an incubation in BFA-free media for 7.5 h (*30 min*) or for 2 h followed by an incubation in BFA-free media for 6 h (*2 hour*). During the last hour of BFA treatment, 2.5 μ M teniposide was added prior to analysis by colony forming assay. The toxicity of teniposide in the absence of stress (*none*) is also shown. Percent control survival was determined for each treatment by dividing the percent survival of BFA-treated cells by the percent survival of solvent-treated cells. Shown are the mean percent control cell survival \pm SEM from 3-5 independent experiments with three replicates per experiment. * - a statistically significant increase in cell survival was observed in BFA-treated cells compared to non-stressed cells (one way ANOVA followed by multiple comparison using Bonferroni's test with P < 0.05); there was no statistically significant difference in cell survival between the 30-min and 2-h BFA exposures.

Figure 9. BFA-induced resistance to teniposide can be reversed with PGA₁ after the induction of stress. EMT6 cells were exposed to 10 µg/mL brefeldin A (B) for 30 min (added at the 0-hour time point) and then incubated in BFA-free media for 7.5 h. During the last hour of stress treatment, 2.5 µM teniposide (T) was added (at the 7-hour time point) prior to analysis by colony forming assay (COL, at 8-hour time point). To determine the time-dependence of reversal, 25 µM prostaglandin A₁ (PGA₁) was added either 30 min before BFA treatment (at the -0.5-hour time point) or after BFA treatment (at the 0.5-hour, 1-hour, 2-hour, 4-hour, or 6-hour time points). Percent control survival was determined by dividing the cell survival of BFA-treated cells by the cell survival of non-treated cells. Values represent the mean percent control cell survival ± SEM from three independent experiments with three replicates per experiment. * - a statistically significant decrease in cell survival was observed in cells treated with PGA₁ prior to, during and up to 7 h after BFA treatment when compared to BFA treatment alone (one way ANOVA followed by multiple comparison using Bonferroni's test with P < 0.05).

Fig 1

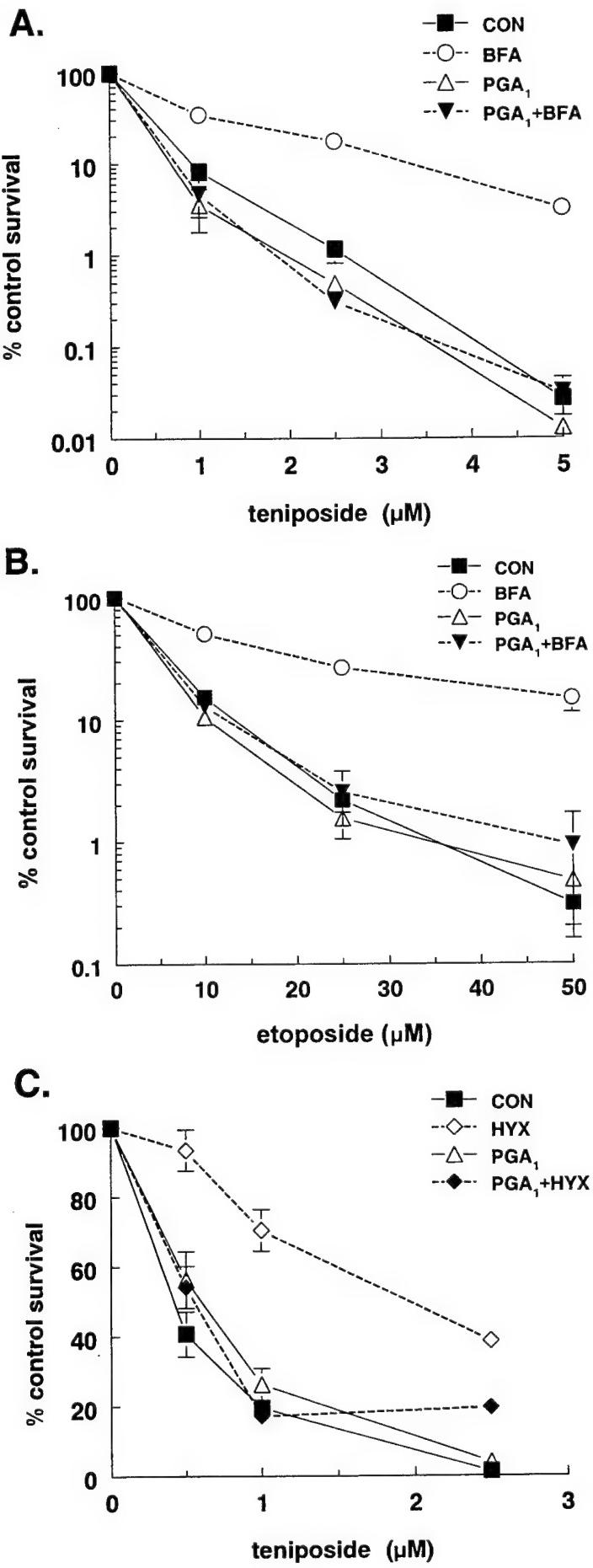


Fig 2

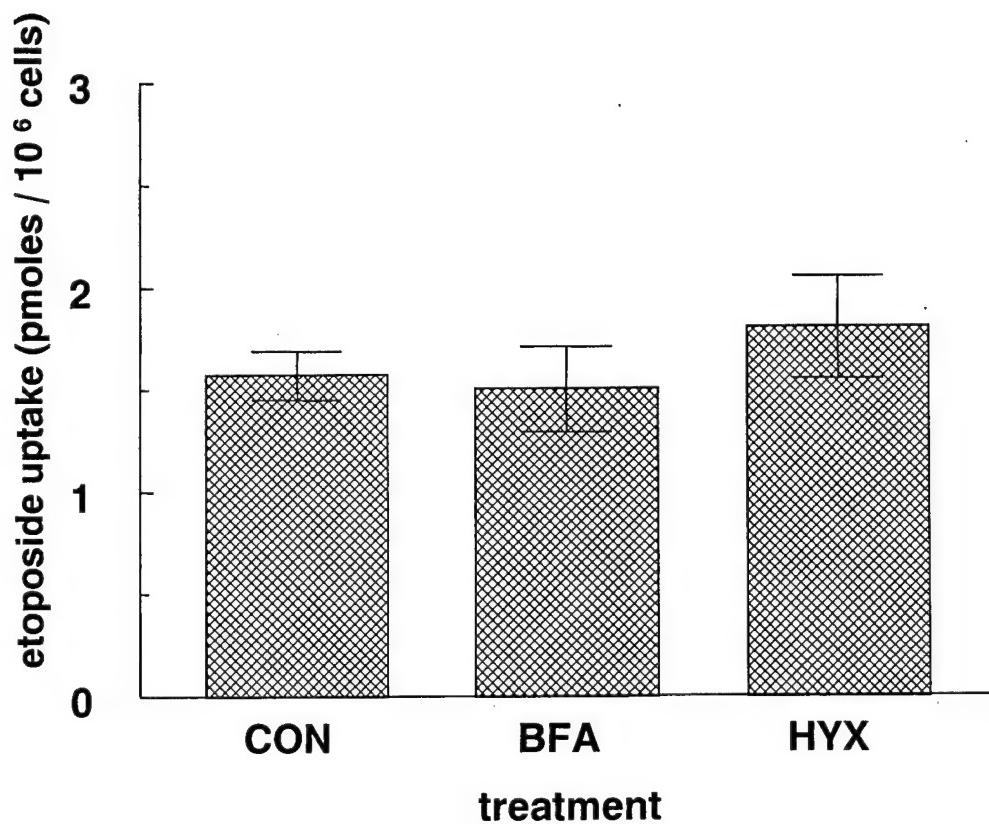


Fig 3A

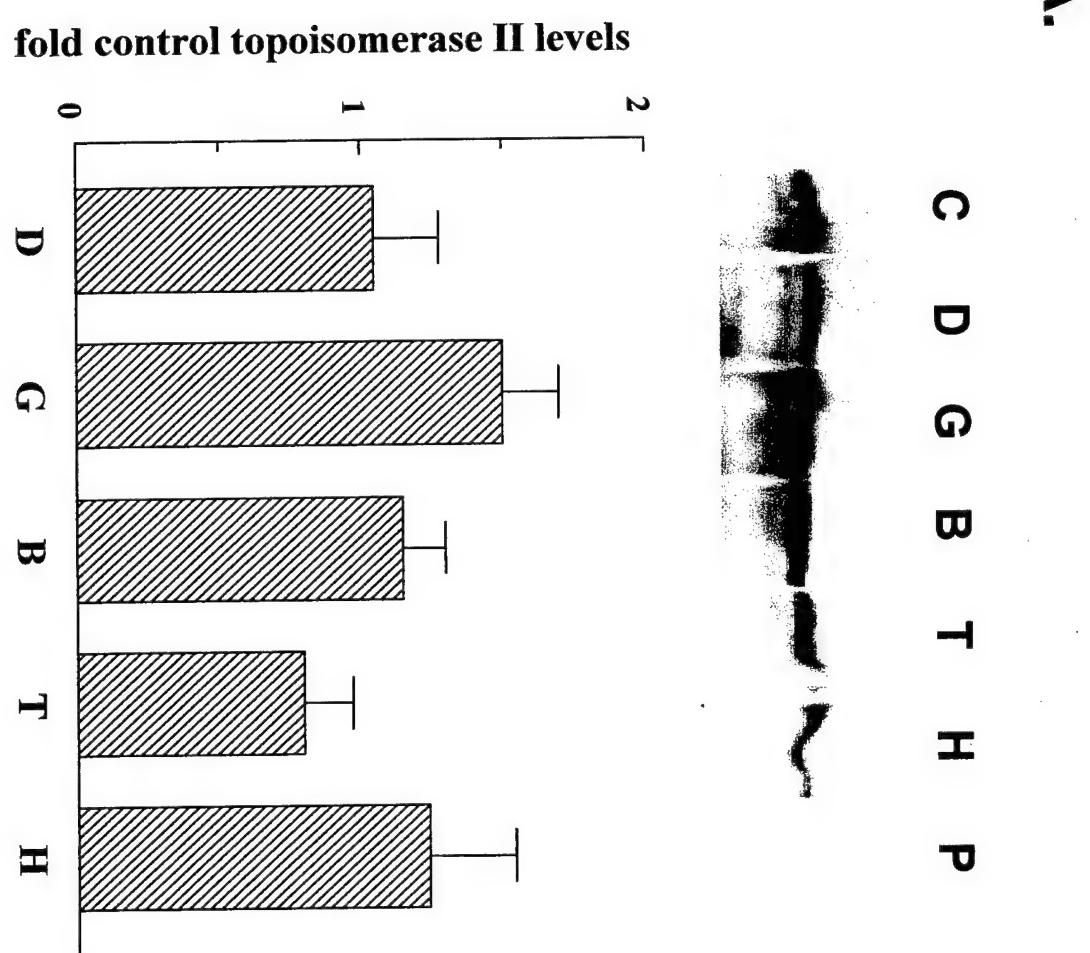


Fig 3B

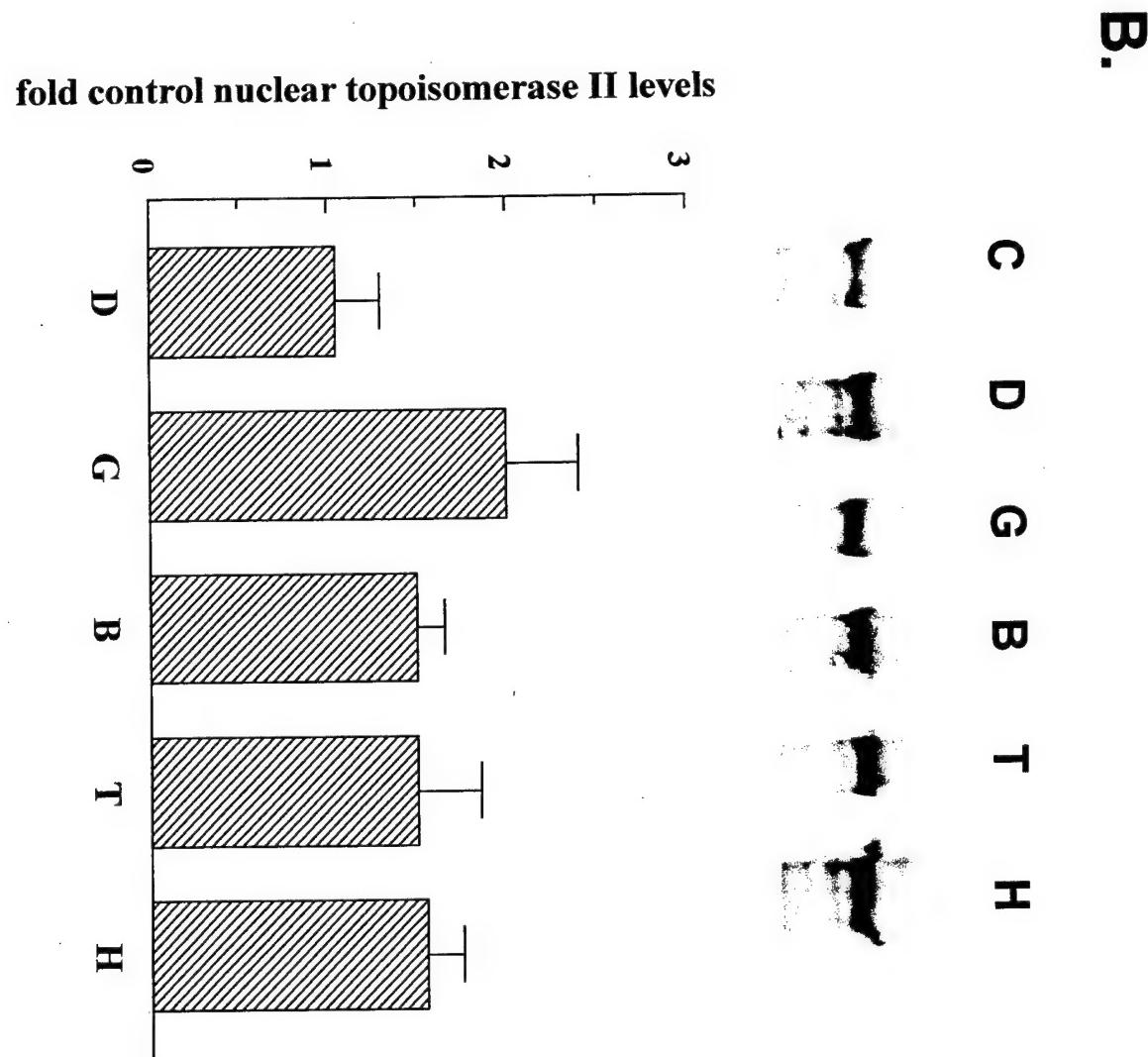
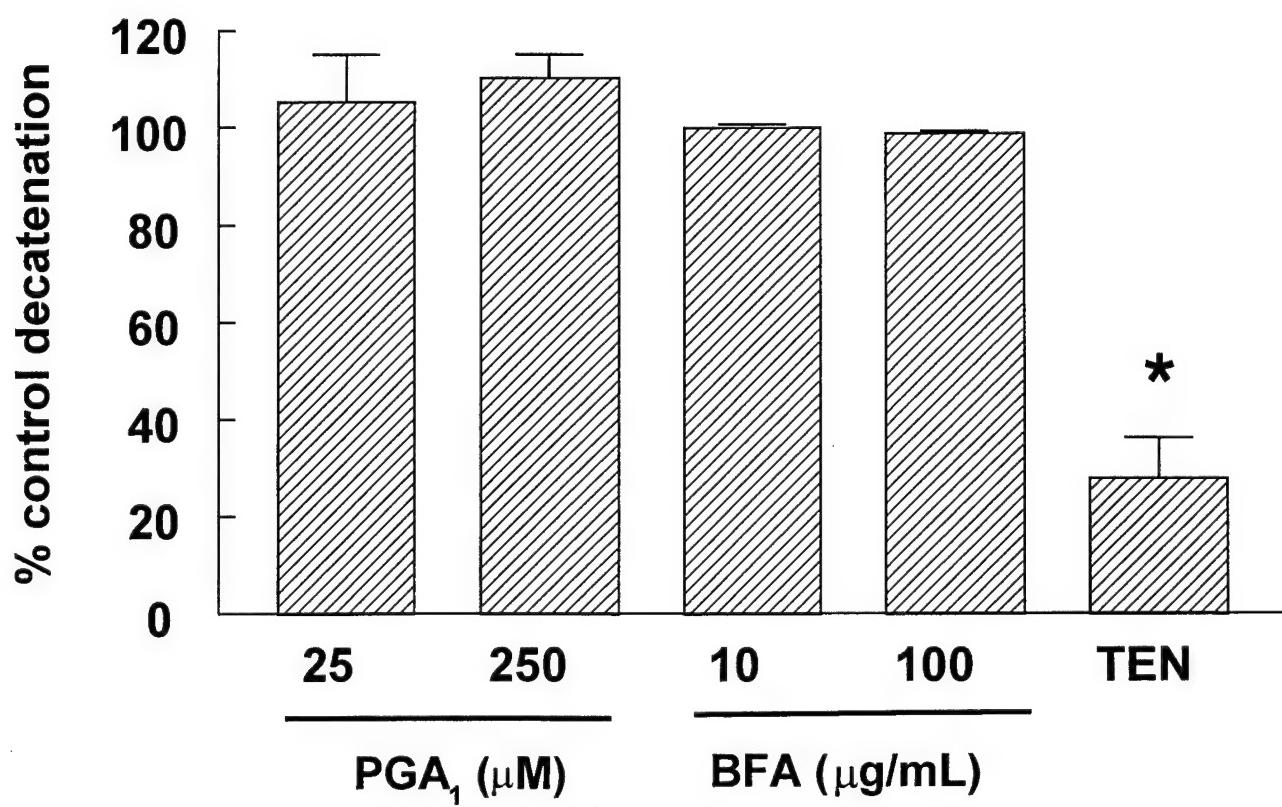


Fig 4



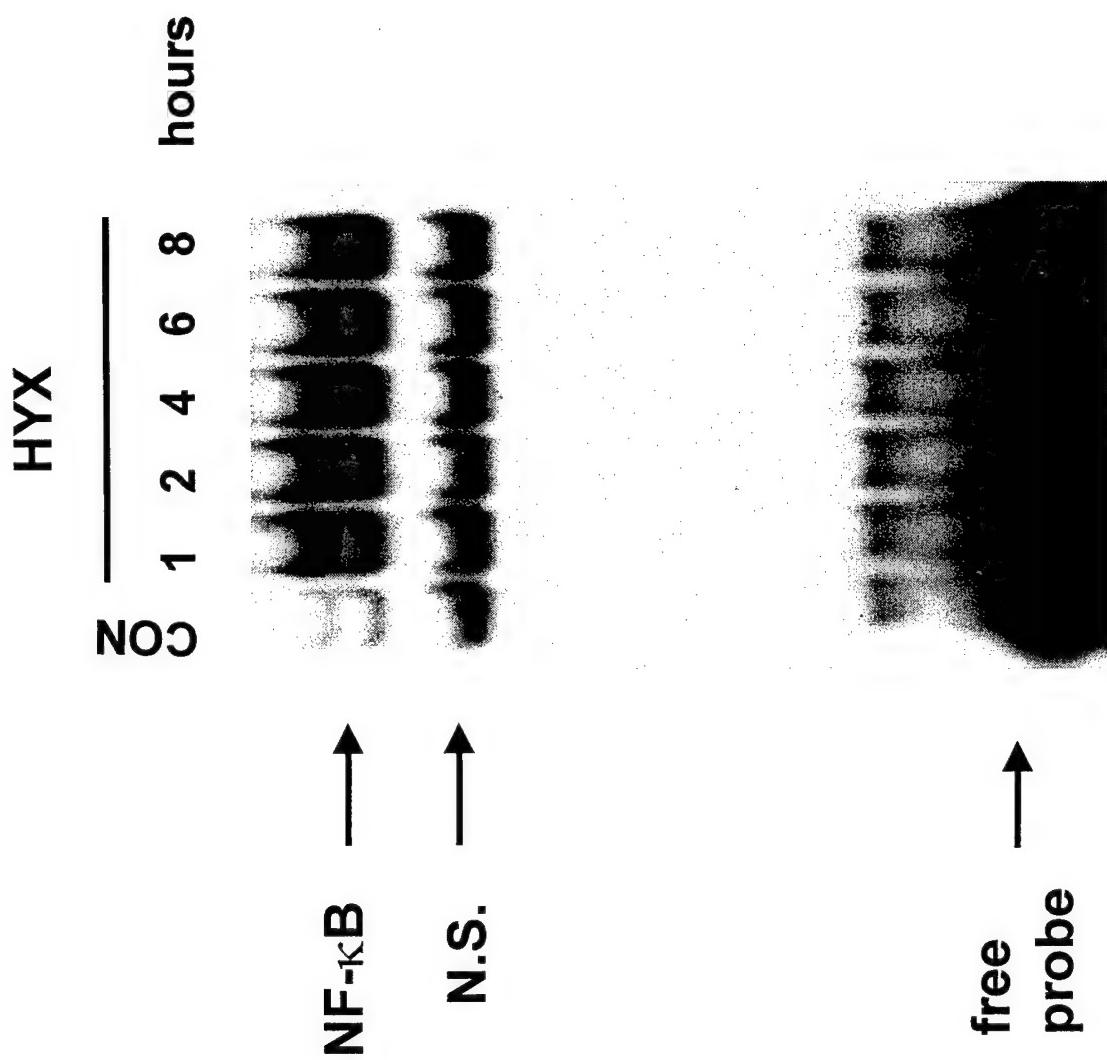


Fig 6A

A.

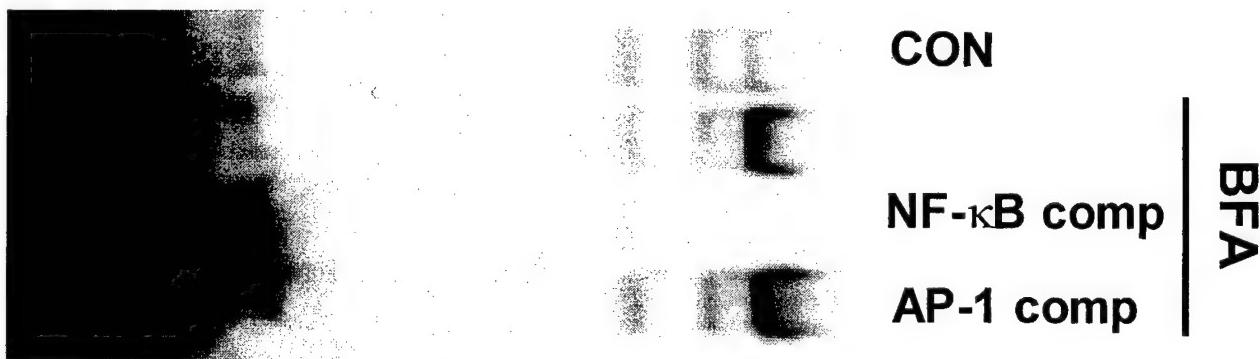
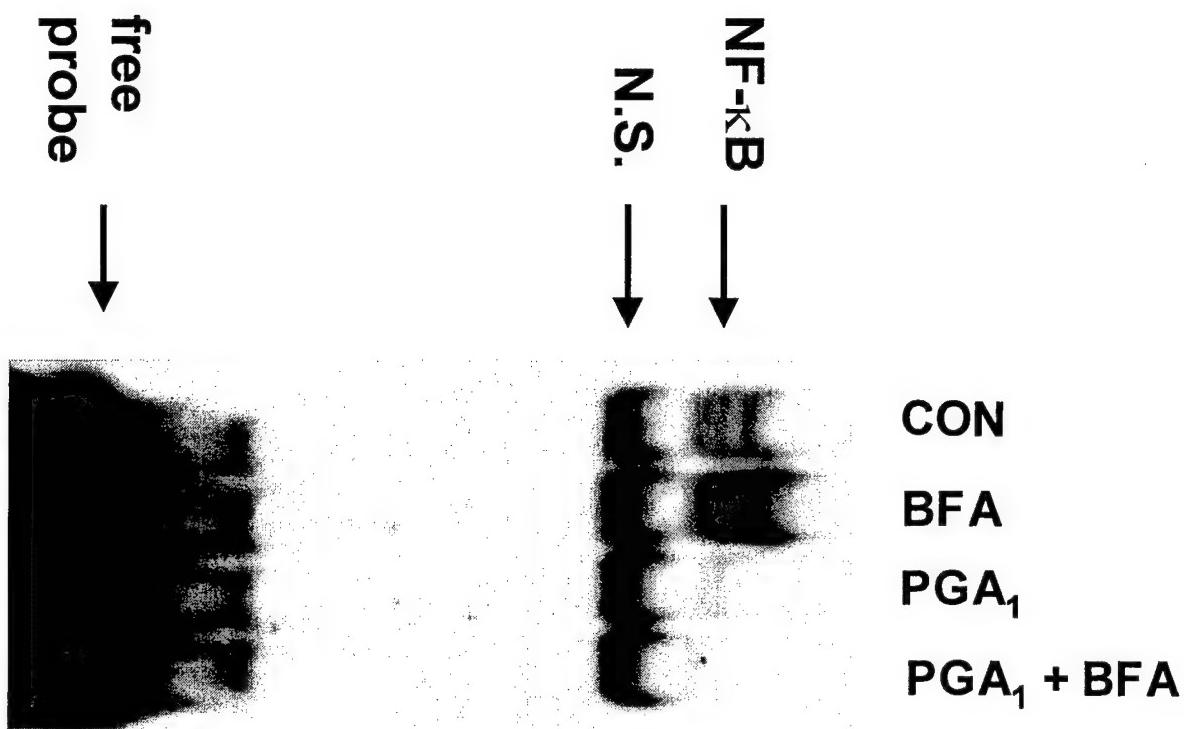


Fig 6B

B.

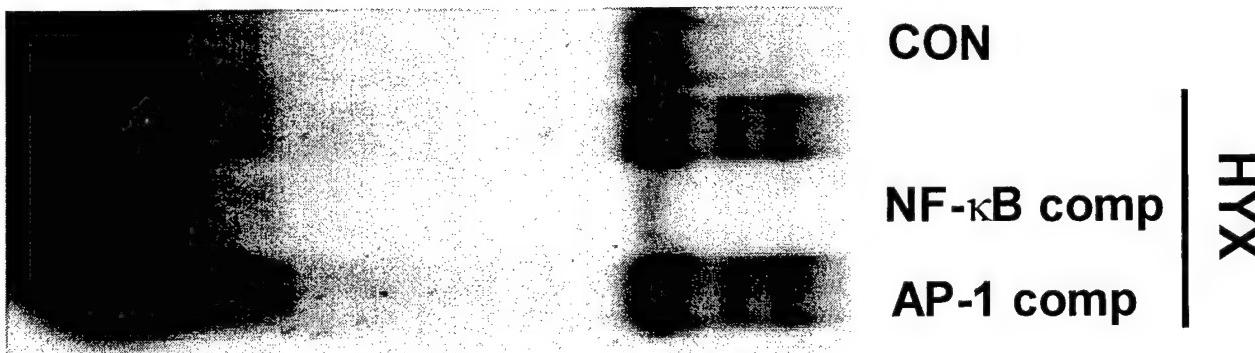
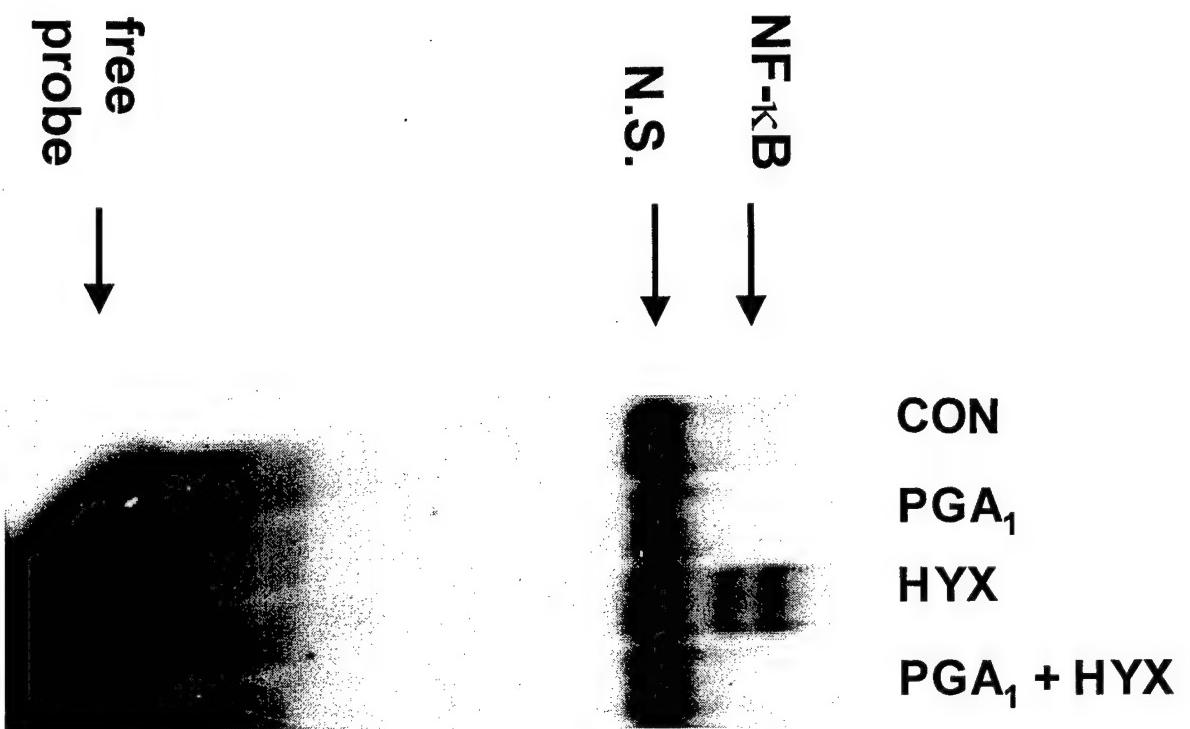


Fig 6C

C.

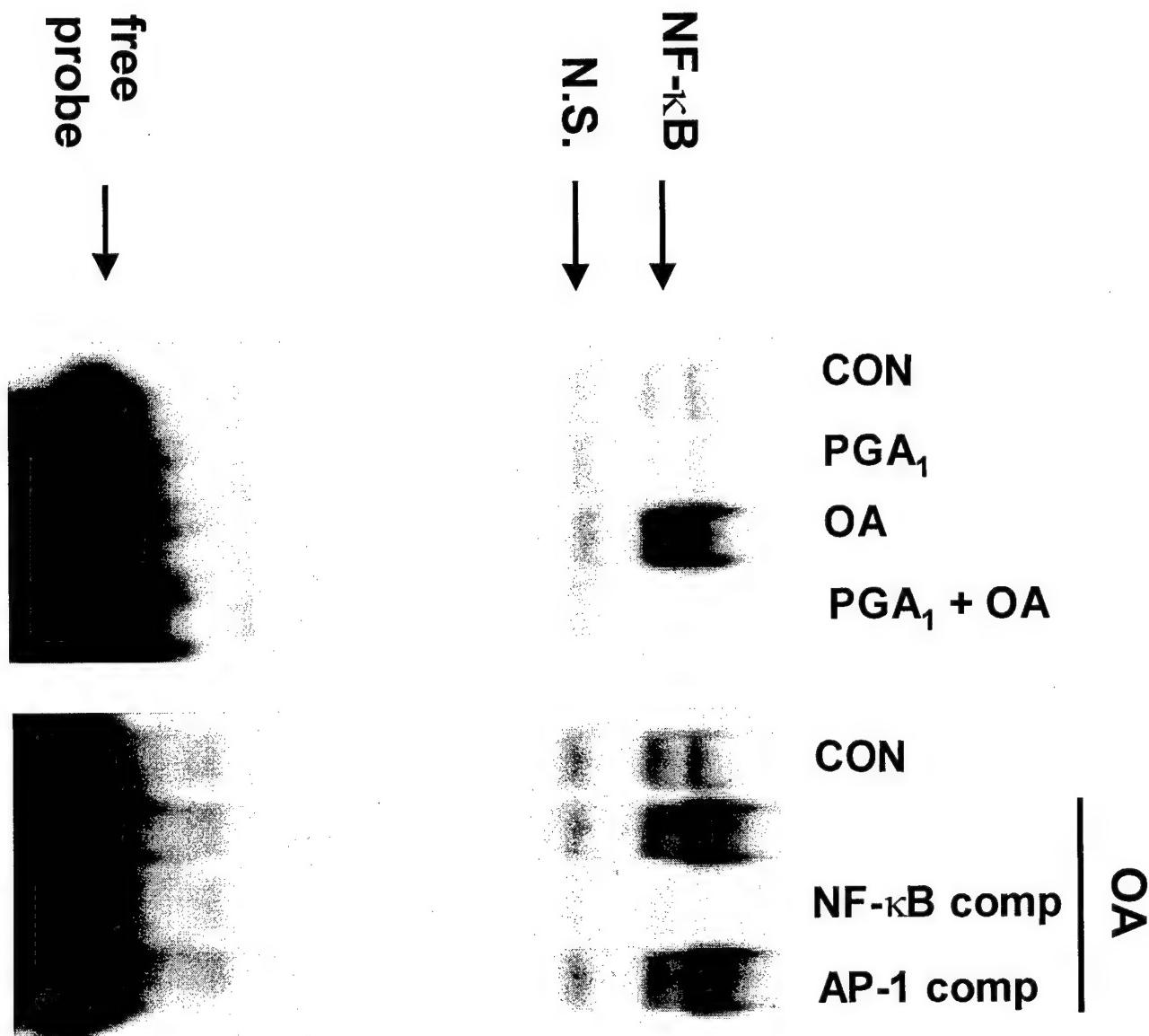


Fig 7

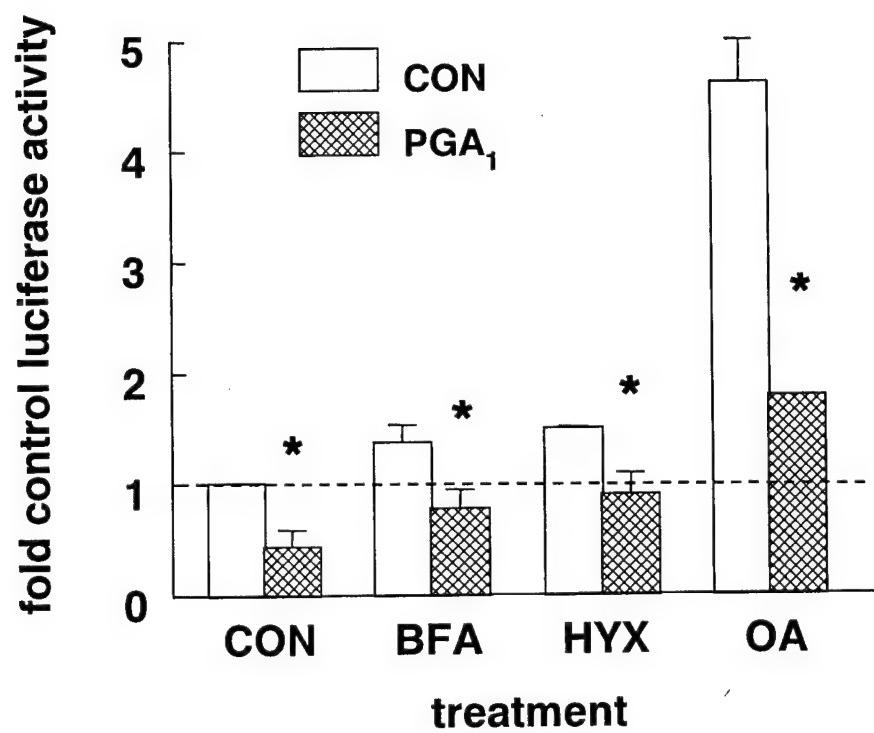


Fig 8

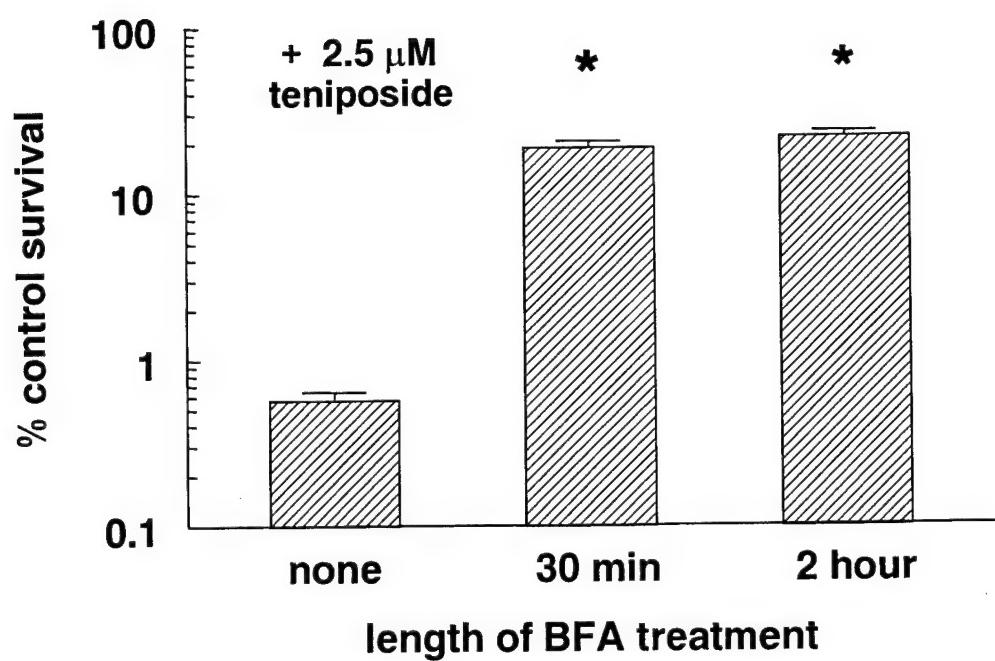
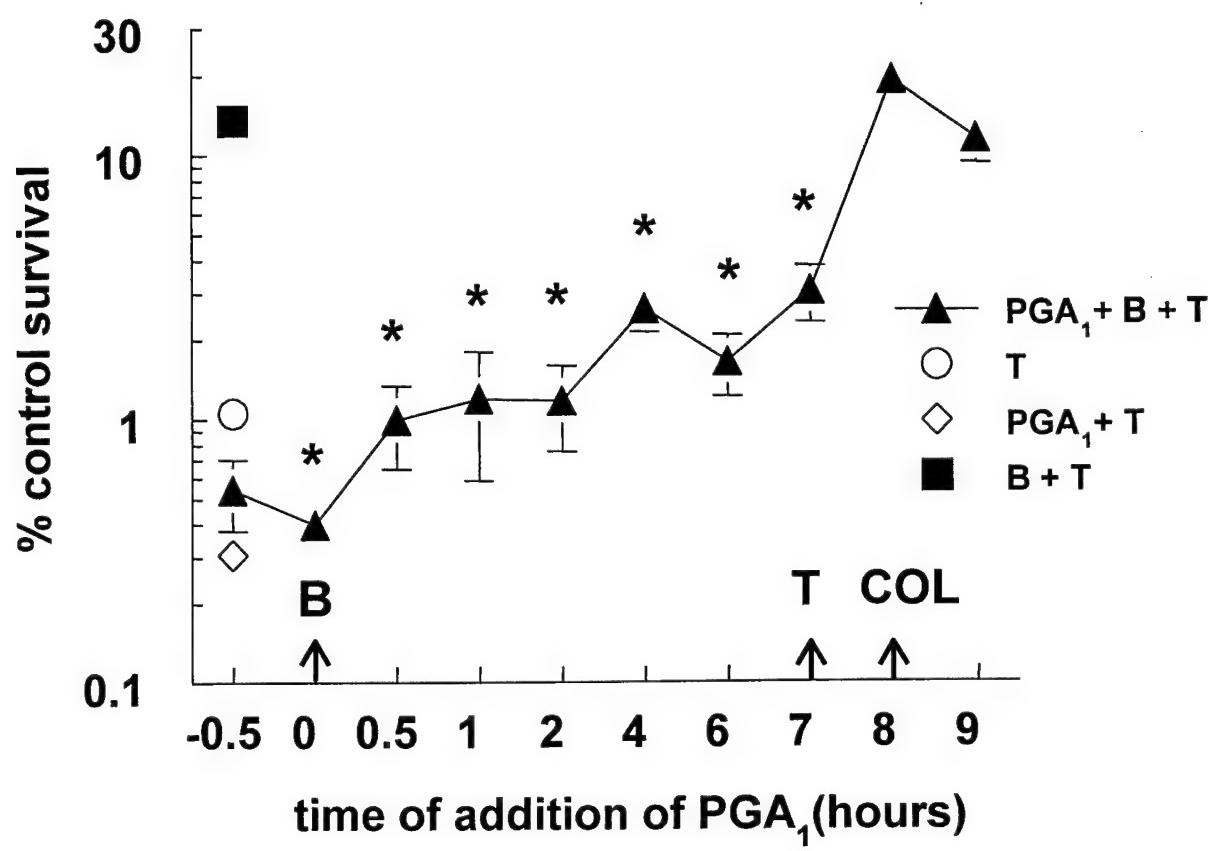


Fig 9



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Inducible Expression of the p65 or p50 NF-κB Subunits Causes Resistance to Topoisomerase II Inhibitors and Insensitivity to Stress

Lori M. Brandes, Steven R Patierno, Katherine A Kennedy, The George Washington University Medical Center, Washington, DC.

One of the major limiting factors to the successful treatment of breast cancer is the development of drug resistance. Our data show that treatment with hypoxia or the chemical stress agent brefeldin A causes resistance of EMT6 mouse mammary tumor cells to anticancer agents that inhibit topoisomerase II. Furthermore, our work suggests the transcription factor NF-κB mediates this form of resistance, as inhibition of NF-κB activation is capable of preventing drug resistance. To determine if activation of NF-κB is sufficient to cause drug resistance, we have expressed the two predominant subunits of NF-κB, p65 and p50, in EMT6 cells using an ecdysone inducible expression system. We now show that expression of p65 or p50 results in enhanced basal levels of NF-κB transactivation and the development of resistance to etoposide. Preliminary results also suggest that p65- and p50-expressing cells are insensitive to stress-induced NF-κB activation and stress-induced drug resistance. Taken together, these data support the role of NF-κB activation in stress-induced drug resistance and imply that inhibition of NF-κB may improve tumor responsiveness to clinical cancer therapy. Supported by Army Breast Cancer Initiative Award #99-1-9186 (K.A.K.)

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Reversal of Stress-Induced Resistance to Topoisomerase II Inhibitors with Inducible Expression of a Dominant Negative Mutant of I_kB α

Lori M Brandes, Dietrich A Stephan, Steven R Patierno, Katherine A Kennedy, The George Washington University Medical Center, Washington, DC; Center for Genetic Medicine, Children's National Medical Center, Washington, DC.

Physiological stress conditions associated with solid tumors are known to cause resistance to anticancer agents. We have shown EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) become insensitive to the topoisomerase II inhibitors etoposide, teniposide, and doxorubicin. Our previous data demonstrate that pretreatment with agents that inhibit NF- κ B activation, such as prostaglandin A1 or the proteasome inhibitor MG-132, prevents hypoxia- and BFA-induced resistance to teniposide, suggesting that NF- κ B mediates stress-induced resistance to topoisomerase II inhibitors. To determine the involvement of NF- κ B in stress-induced drug resistance, we have introduced an inducible dominant negative mutant expression construct (I_kB α M) into EMT6 cells to selectively block NF- κ B activation. Our data show that I_kB α M expression inhibits stress-induced NF- κ B activation and reverses both hypoxia- and BFA-induced resistance to etoposide and doxorubicin. Furthermore, the extent of reversal of drug resistance is dependent upon the extent of induction of I_kB α M expression. Efforts are underway to further elucidate the mechanism of stress-induced drug resistance through NF- κ B activation using gene microarray analysis of stress-treated I_kB α M cells. Supported by Army Breast Cancer Initiative Award #99-1-9186 (K.A.K.) and by an endowment fellowship from the Washington Metropolitan Chapter of the Achievement Rewards for College Scientists (L.M.B.).

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**STRESS-INDUCED RESISTANCE OF BREAST TUMOR CELLS TO
TOPOISOMERASE II INHIBITORS IS MEDIATED BY NF-κB ACTIVATION**

Lori M. Brandes, Steven R. Patierno, Dietrich A. Stephan, and Katherine A. Kennedy.
The George Washington University Medical Center, Washington DC, 20037; Research Center for Genetic Medicine, Children's National Medical Center, Washington DC, 20010. Clinical success of breast cancer chemotherapy is often limited by the development of drug resistance. Recently a form of intrinsic drug resistance that develops in response to physiological stress conditions associated with solid tumors has been identified. We have shown that treatment of EMT6 mouse mammary tumor cells with the physiological stress, hypoxia, or the chemical stress, brefeldin A (BFA), causes resistance to the topoisomerase II (topoII) inhibitors etoposide and doxorubicin. Stress treatment with hypoxia or BFA is known to cause the activation of numerous stress-responsive proteins, including the nuclear transcription factor NF-κB. To determine the involvement of NF-κB in stress-induced drug resistance, we have introduced an inducible dominant negative mutant of I κ B α (I κ B α M, S32/36A) into EMT6 cells to selectively block NF-κB activation. Our data show that expression of I κ B α M inhibits BFA- and hypoxia-induced NF-κB activation and prevents the development of stress-induced drug resistance. Moreover, the extent of reversal of stress-induced drug resistance is dependent upon the extent of induction of I κ B α M. To test if NF-κB activation is sufficient to cause drug resistance, we have expressed the two predominant subunits of NF-κB, p65 and p50, in EMT6 cells. Inducible expression of p65 or p50 results in enhanced basal levels of NF-κB transactivation and the development of resistance to etoposide and doxorubicin. To further elucidate the mechanism of stress-induced resistance through NF-κB activation, we have performed gene expression analysis of stress-treated I κ B α M cells using the Affymetrix GeneChip®. Our results suggest that stress-treated I κ B α M cells do not develop resistance to topoII inhibitors because they exhibit altered expression of apoptosis-related genes when compared to stress-treated vector-transfected cells that are drug resistant. Taken together, our findings support the involvement of NF-κB activation in stress-induced resistance to topoII inhibitors and imply activation of NF-κB by stress results in down-regulation of genes responsible for drug-dependent cytotoxicity. These data imply that inhibition of NF-κB activation in tumors could enhance the clinical efficacy of anticancer drugs. Supported by Army Breast Cancer Initiative Award #99-1-9186 (K. A. K.) and by an endowment fellowship from the Washington Metropolitan Chapter of the Achievement Rewards for College Scientists (L. M. B.).

Reversal of Stress -induced resistance to topoisomerase II inhibitors with
inducible expression of a dominant negative mutant of $\text{IkB}\alpha$

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Abstract

Physiological stress conditions associated with solid tumors are known to cause resistance to anticancer agents. We have shown EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) become insensitive to the topoisomerase II inhibitors etoposide, teniposide, and doxorubicin. Our previous data demonstrate that pretreatment with agents that inhibit NF- κ B activation, such as prostaglandin A₁ or the proteasome inhibitor MG-132, prevents hypoxia- and BFA-induced resistance to teniposide, suggesting that NF- κ B mediates stress-induced resistance to topoisomerase II inhibitors. To determine the involvement of NF- κ B in stress-induced drug resistance, we have introduced an inducible dominant negative mutant expression construct ($I\kappa B\alpha M$) into EMT6 cells to selectively block NF- κ B activation. Our data show that $I\kappa B\alpha M$ expression inhibits stress-induced NF- κ B activation and reverses both hypoxia- and BFA-induced resistance to etoposide and doxorubicin. Furthermore, the extent of reversal of drug resistance is dependent upon the extent of induction of $I\kappa B\alpha M$ expression. Efforts are underway to further elucidate the mechanism of stress-induced drug resistance through NF- κ B activation using gene microarray analysis of stress-treated $I\kappa B\alpha M$ cells. Supported by Army Breast Cancer Initiative Award #99-1-9186 (K.A.K.) and by an endowment fellowship from the Washington Metropolitan Chapter of the Achievement Rewards for College Scientists (L.M.B.)

Introduction

Most solid tumors are resistant to conventional cancer chemotherapies. Clinical drug resistance is caused, in part, by physiological stresses associated with the solid tumor microenvironment such as low pH, glucose deprivation, and hypoxia. Tumor drug insensitivity can be reproduced in the laboratory with chemical stress agents that mimic biological stress. We have previously shown that treatment with the chemical stress, brefeldin A (BFA), or the physiological stress, hypoxia, causes EMT6 mouse mammary tumors to develop resistance to the topoisomerase II (topoll) inhibitors etoposide and doxorubicin. Stress treatment with hypoxia or BFA is known to activate numerous stress-responsive proteins, including the nuclear transcription factor NF- κ B. To test if NF- κ B activation is sufficient to cause drug resistance, we expressed the two predominant subunits, p65 and p50, in EMT6 cells. Inducible expression of P65 or p50 results in enhanced NF- κ B transactivation and the development of resistance to etoposide and doxorubicin. To determine role of NF- κ B in stress-induced drug resistance, we have introduced a mutant inhibitor of NF- κ B, I κ B α (I κ B α M, S32/36A), into EMT6 cells to selectively block NF- κ B activation. To further elucidate the mechanism of stress-induced resistance through NF- κ B activation, we have performed gene expression analysis of stress-treated I κ B α M cells using the Affymetrix GeneChip®. Taken together, our findings support the involvement of NF- κ B activation in stress-induced resistance to topoll inhibitors and imply activation of NF- κ B by stress results in down-regulation of genes responsible for drug-dependent cytotoxicity. These data imply that inhibition of NF- κ B activation in tumors could enhance the clinical efficacy of anticancer drugs.

Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells (provided by Dr. S. Rockwell, Yale University) were grown in Waymouth's MB 752/1 medium (GibcoBRL, Grand Island, NY) supplemented with L-glutamine (Life Technology, Gaithersburg, MD), 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25 µg/ml gentamicin sulfate (Biofluids, Rockville, MD) as described previously (Lin et al., 1998).

Reagents and Treatments. Brefeldin A (Sigma, St. Louis, MO) was dissolved in 70% ethanol to a concentration of 10 mg/ml and stored at 4°C. For BFA treatment, cells were exposed to 10 µg/ml BFA for 2 h and then incubated 2-6 h in BFA-free media. For hypoxia treatment, cells were grown in 75cm² glass flasks for 48 h. Thereafter, flasks were fitted with a rubber Suba seal with a 18 gauge, 1" needle inlet and a 18 gauge, 1.5" needle outlet, and then gassed with a humidified mixture of 95% N₂, 5% CO₂ (certified O₂<0.05ppm) at 37°C for 2-8 h. Etoposide (Sigma, St. Louis, MO), 100 µM in DMSO, was stored at -20°C. Cells were treated with 10-50 µM etoposide 1 h prior to clonogenicity assay. Ponasterone A (Invitrogen, San Diego, CA) was rehydrated in 70% ethanol to a concentration of 10 mM and stored at -20°C. Cells were treated with 0.1-10 µM ponasterone A for 24 hours to obtain expression of IκBαM.

Western blot analysis. Cells were seeded in 100 mm² dishes and incubated under maintenance conditions for 24 hours. After treatment with ponasterone A, cells were washed in cold PBS, lysed in 100 µl of 1X SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.006% bromophenol blue), and boiled for 5 min. The protein concentrations of the samples were determined using the BCA method (Pierce, Rockford, IL). Protein samples (20-100 µg) were mixed with 2X SDS (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, 2% β-mercaptoethanol) and boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel (4% stacking gel, pH 6.8, 10% resolving gel, pH 8.8, 30: 0.8 acrylamide:bisacrylamide) at 120 Volts for 2 h in a running buffer containing 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The resulting gel was transferred to a nitrocellulose membrane by electrophoresis for 45 min at 55 Volts in 1X CAPS buffer (10 mM CAPS, pH 11). Following transfer, the membrane was incubated in 1X TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) with 1% bovine serum albumin. To detect IκBαM-HA expression, western blots from nontransfected EMT6, VCT, and IκBαM cells treated with ponasterone A were probed with an anti-HA antibody (Sigma, St. Louis) diluted 1000-fold in 1X TBST with 1% BSA overnight at 4°C. The membrane was then washed with 1X TBST and incubated with an HRP-conjugated IgG anti-rabbit antibody (1:10000 dilution in 1X TBST with 1% BSA) for 1 h at room temperature. The membrane was washed again and antibody binding was visualized with enhanced chemiluminescent reagent (Pierce, Rockford, IL).

Luciferase reporter gene assay. Cells were transfected with 2.5 ml of serum-free Waymouth's media containing 3 µg of the luciferase reporter plasmid and 1 µg of pcDNA3.1-lacZ (Invitrogen, San Diego, CA) in 12 µl TransFast reagent (Promega, Madison, WI). Following drug treatments, cells were washed in cold PBS and lysed for 15 min at room temperature in 400 ul of Reporter Lysis Buffer (Promega, Madison, WI). Cell debris was removed by centrifugation at 10,000 rpm for 2 min at 4°C. For the luciferase assay, 100 µl of Luciferase Assay Reagent containing luciferol (Promega, Madison, WI) was added to 20 µl of cell lysate. Light emission was measured using a Beckman scintillation counter using the single photon monitor mode over a 1 min interval. Cells were also assayed for lacZ expression to correct for differences in transfection efficiency. Cell lysate (100-150 µl) was mixed with 150 µl of assay buffer containing o-nitrophenyl-β-D-galactopyranoside and incubated for 2 h at 37°C. The OD at 420 nm was measured using a spectrophotometer and the relative β-galactosidase activity for each sample was used to normalize luciferase activities.

EMSA. Electrophoretic mobility shift assays were performed as previously described (Lin et al., 1998). Briefly, cells were seeded at a density of 3-4 X 10⁴ cells/ml in 150 mm² dishes or 150 cm² glass flasks. Following drug treatment, cells were washed in cold PBS and lysed in 100 µl lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40, 0.5 mM sodium orthovanadate, 1 mM PMSF) on ice for 5 min. Cell nuclei were separated by centrifugation at 5,000 rpm for 5 min at 4°C and then washed with 500 µl washing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5 mM sodium orthovanadate, 1 mM PMSF). The nuclei were then broken by three freeze-thaw cycles in an ethanol-dry ice bath. Protein concentration was determined using the micro BCA method (Pierce, Rockford, IL). To construct the probe, 3.5 pmole of oligonucleotide containing the NF-κB consensus sequence (Promega, Madison, WI) was incubated with 1 µl of [γ -³²P] ATP (10 mCi/ml, 6,000 Ci/mmol, Amersham-Pharmacia, Arlington Heights, IL), 5 units of T4 polynucleotide kinase (Promega, Madison, WI) and 10 µl of end-labeling buffer at 37°C for 1 h. The reaction was terminated with 90 µl 1X STE buffer (Sigma, St. Louis, MO) and then passed through a G-25 spin column (Worthington Biochem, Lakewood, NJ). Nuclear protein extracts (15-20 µg) were incubated with 3 µg poly dI-dC and 0.035 pmole of radiolabeled oligonucleotide (100,000-200,000cpm) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 0.5 mM dithiothreitol, 4% glycerol) at room temperature for 20 min. The binding mixtures were loaded on a non-denaturating 6% polyacrylamide gel (30:1 acrylamide:bisacrylamide, 0.5 X TBE, 2.5% glycerol) and separated in 0.5 X TBE buffer at 110 Volts for 3 h. The resulting gel was transferred to filter paper and dried under vacuum pressure in a gel dryer (Model 583, BioRad, Richmond, CA). After drying, the gel was exposed to X-ray film at -80°C in a cassette with intensifying screens.

Colony forming assay. EMT6 cells were seeded in 25 cm² tissue culture flasks and incubated under maintenance conditions for 24-40 hours prior to treatment. Following treatment, cells were harvested with trypsin, counted and serially

diluted in Waymouth media. Cells were seeded in 60 mm² triplicate tissue culture dishes and incubated under maintenance conditions for 7 d. Colonies were stained with 0.25% crystal violet and counted as previously described (Lin et al., 1998).

Affymetrix GeneChip™ Expression Analysis. Cells were seeded at a concentration of 8 X 10⁴ cells/ml in 75 cm² glass flasks and incubated under maintenance conditions for 24 hours. Following drug and stress treatments, total RNA was collected using TRIzol reagent (Sigma, St. Louis, MO). Double-stranded cDNA was synthesized from total RNA samples using the SuperScript™ Choice System (GibcoBRL, Rockville, MD). For cDNA cleanup, 162 µl of (25:24:1) phenol:chloroform:isoamyl alcohol saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (Sigma, St. Louis, MO) was added and the samples were transferred to Phase Lock Gel PLG I-light tubes (Eppendorf, Westbury, NY). After centrifugation at 14,000g for 2 minutes, the upper aqueous phase was isolated and 0.5 volumes of cold 7.5 M NH₄Ac (Sigma, St. Louis, MO) and 2.5 volumes of absolute ethanol (Sigma, St. Louis, MO) were added. cDNA was pelleted by centrifugation at 14,000g for 20 minutes at 4°C. The resulting pellets were washed twice with 0.5 ml of cold 80% ethanol, air-dried, and resuspended in 12 µl nuclease-free water. Labeled cRNA was synthesized using the ENZO BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, distributed by Affymetrix, Santa Clara, CA). cRNA samples (20 µg) were fragmented by incubation in 8 µl of 5X fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 minutes. To create hybridization cocktails, cRNA (15 µg) was mixed with 5 µl of 3 nM Control Oligonucleotide B2 (Affymetrix, Santa Clara, CA), 15 µl of 20X Eukaryotic Hybridization Controls (Affymetrix, Santa Clara, CA), 3 µl of 10 mg/ml Herring sperm DNA (Fisher, Pittsburgh, PA), 3 µl of 50mg/ml acetylated bovine serum albumin (GibcoBRL, Rockville, MD), and 150 µl of 2X Hybridization Buffer [200 mM MES (Sigma, St. Louis, MO), 2M (Na⁺), 40 mM EDTA, 0.02% Tween 20]. In preparation for hybridization, cocktails were incubated at 99°C for 5 minutes and then at 45°C for 5 minutes. Target RNA quality was first assessed using Test2 Probe Array Chips (Affymetrix, Santa Clara, CA). After analysis on a test chip, hybridization cocktails were incubated on Mu11K murine genomic probe arrays (Affymetrix, Santa Clara, CA). Hybridization cocktail (200 µl) was incubated on Mu11K gene chips for 16 hours at 45°C with rotation (60 cycles/minute). Following incubation, Mu11K arrays were washed and stained using the Affymetrix GeneChip Fluidics Station 400 using the standard array format EukGE-WS2 protocol. In brief, arrays were washed 10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C, washed 4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C, stained for 10 minutes in 600 µl of SAPE stain, washed 10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C, stained for 10 minutes in 600 µl of antibody solution (100 mM MES, 1M [Na⁺], 0.05% Tween 20, 0.005% antifoam, 2 mg/ml acetylated BSA, 0.1 mg/ml normal goat IgG, and 3 µg/ml biotinylated antistrepavidin antibody) at 25°C, stained for 10 minutes in 600 µl of SAPE stain at 25°C, and washed for 15 cycles of 4 mixes/cycle with Wash Buffer

A at 30°C. Arrays were scanned twice using an argon-ion laser at a wavelength of 570 nm (Affymetrix, Santa Clara, CA).

Statistics. For colony forming assays, the results shown are the average percent control survival rates \pm SEM from 3-5 independent experiments with three replicates per experiment. Statistically significant changes in these data were determined using one way ANOVA analysis with multiple comparisons performed using Dunnett's test using $p < 0.05$ (Motulsky, 1995).

Figure 1. Stable I_KB_αM expression in EMT6 cells. EMT6 cells were transfected with a 1:5 ratio of pIND: pVgRXR (vector transfected cells-VCT) or 1:5 ratio of pIND-I_KB_αM: pVgRXR (I_KB_αM). Following antibiotic selection, cells were seeded for protein collection and treated with 10μM ponasterone A for 24 hours. Whole cell lysates were collected and analyzed by western blot with anti-I_KB_α, anti-HA, and anti-actin primary antibodies.

Figure 2. Effects of I_KB_αM expression on stress-induced NF-κB activation. Vector-transfected (VCT) and I_KB_αM cells were treated with 10μM ponasterone A (PON) for 24 hours followed by stress treatment of either 60nM okadaic acid (OA) for 8 hours, 10μg/mL brefeldin A (BFA) for 4 hours, or hypoxia (HYP) for 2 hours. Nuclear extracts were collected and analyzed by gel shift assay.

Figure 3. Toxicity profile of stably-transfected EMT6 cells. EMT6 cells, vector-transfected cells (VCT), and I_KB_αM cells were treated with etoposide one hour prior to clonogenicity assay. Results are percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments. Survival of transfected cells with BFA, HYP or PON treatment was also not significantly different from that of EMT6 cells.

Figure 4. Effects of I_KB_αM expression on stress-induced resistance. Vector-transfected cells (VCT) and I_KB_αM cells were treated with 10μM ponasterone A (PON) for 24 hours and a stress treatment of either **A.** 10μg/mL brefeldin A (BFA) or **B.** hypoxia (HYP) for 8 hours. Etoposide was added one hour prior to clonogenicity assay. Results are percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments. * - a statistically significant decrease in cell survival was seen in I_KB_αM cells treated with PON when compared to I_KB_αM not treated with PON ($p<0.05$, t-test). # - a statistically significant difference was NOT seen in the cell survival of stress-treated I_KB_αM cells treated with PON when compared to nonstressed-I_KB_αM treated only with etoposide.

Stable Expression of $I\kappa B\alpha M$ in EMT6 cells

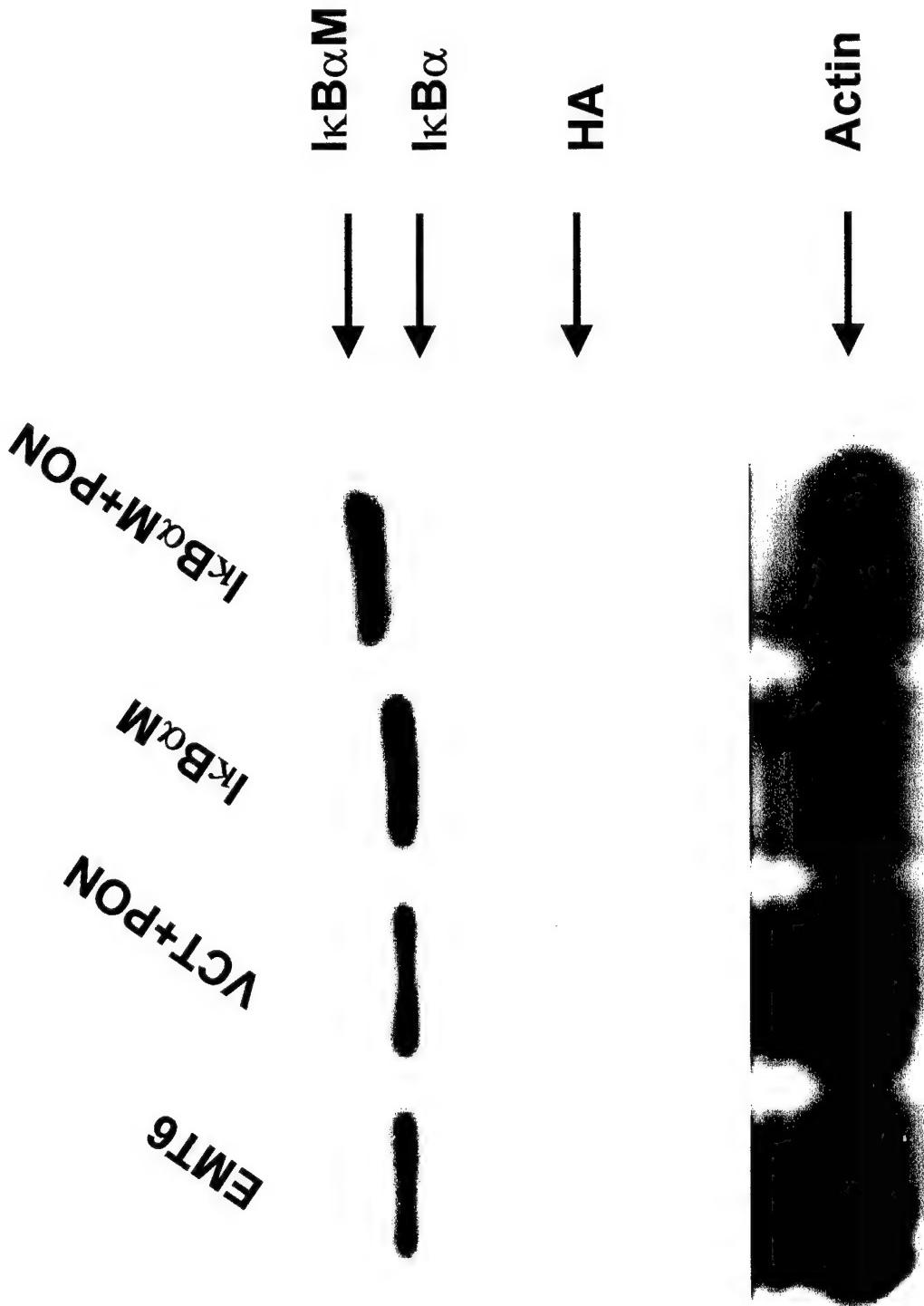


Fig 1

$\text{I}\kappa\text{B}\alpha\text{M}$ Inhibits BFA- and OA-Induced NF- κ B Activation

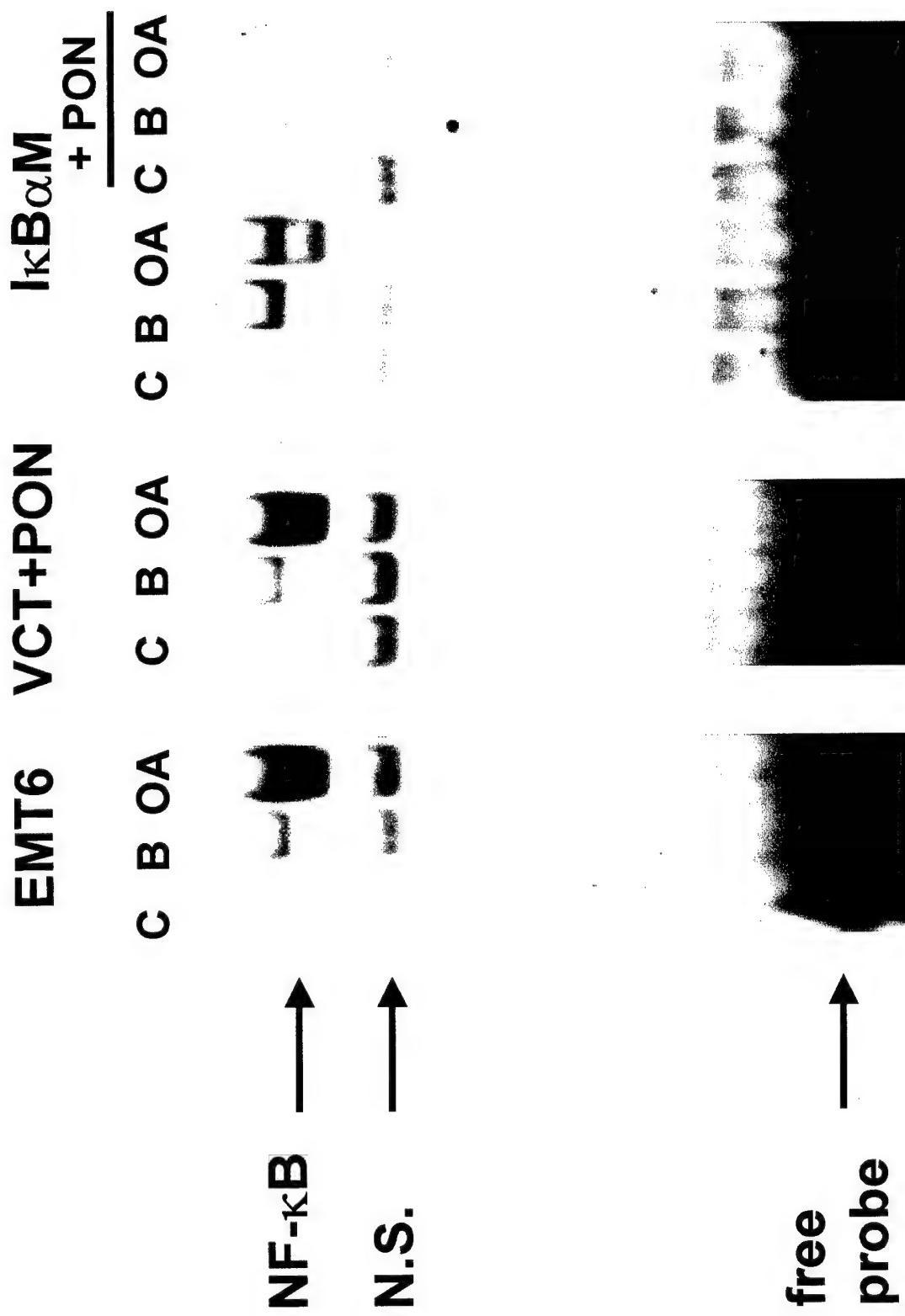


Fig 2

$I\kappa B\alpha M$ Inhibits Hypoxia-Induced NF- κB Activation

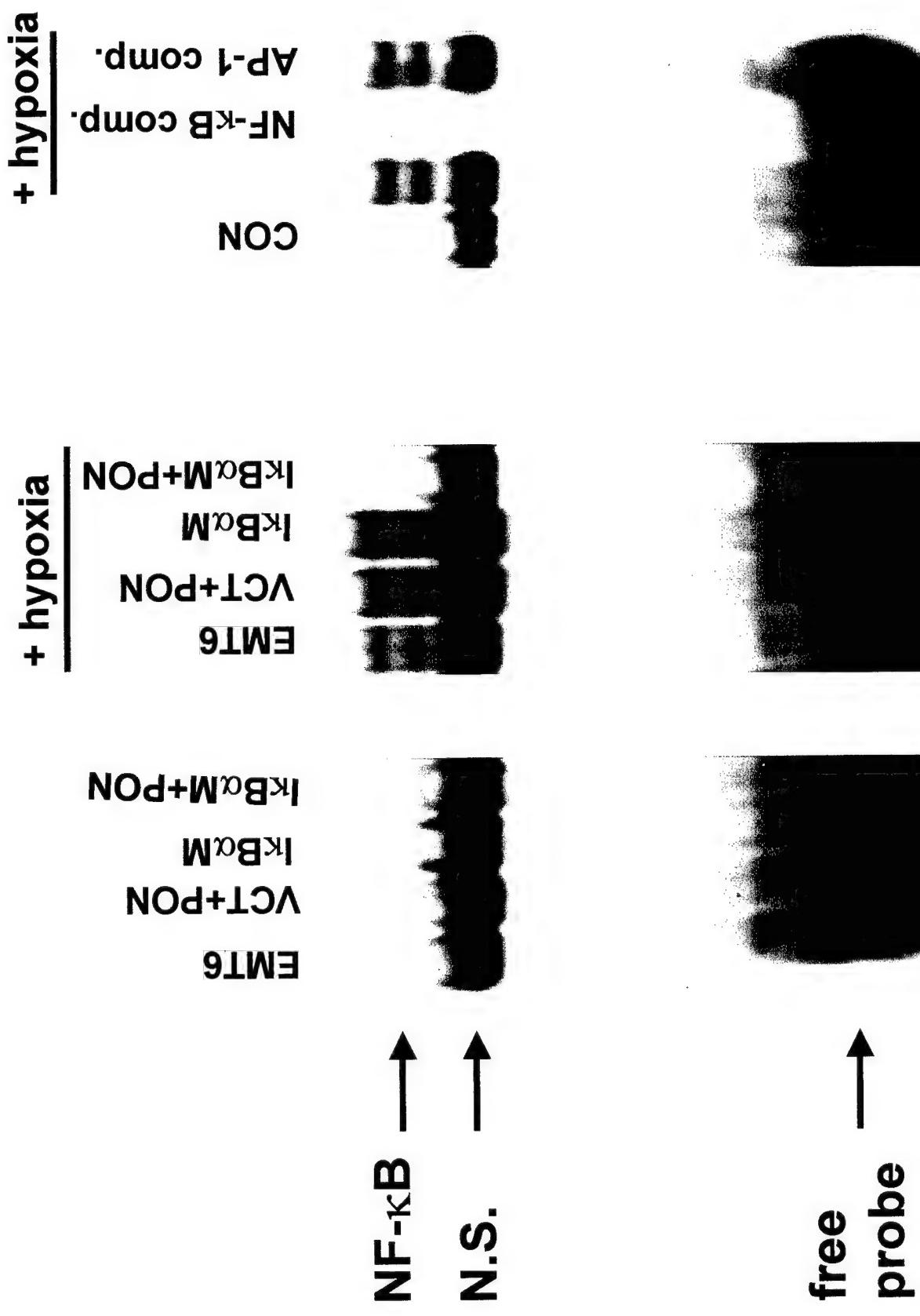


Fig 2 (contd)

Stable Transfection Does Not Alter Etoposide Cytotoxicity

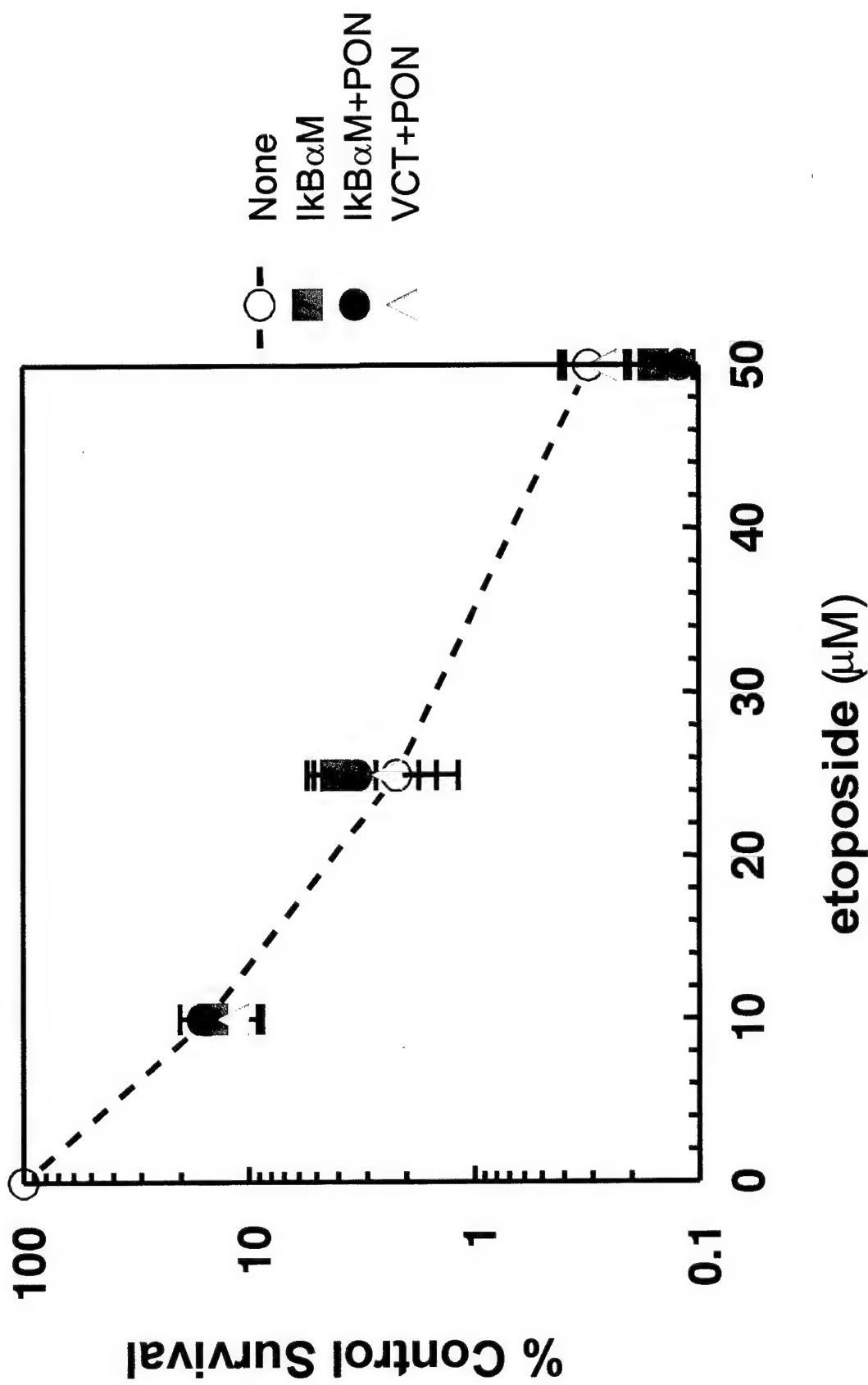


Fig 3

A. Expression of $\text{I}\kappa\text{B}\alpha\text{M}$ Reverses
BFA-Induced Resistance to Etoposide

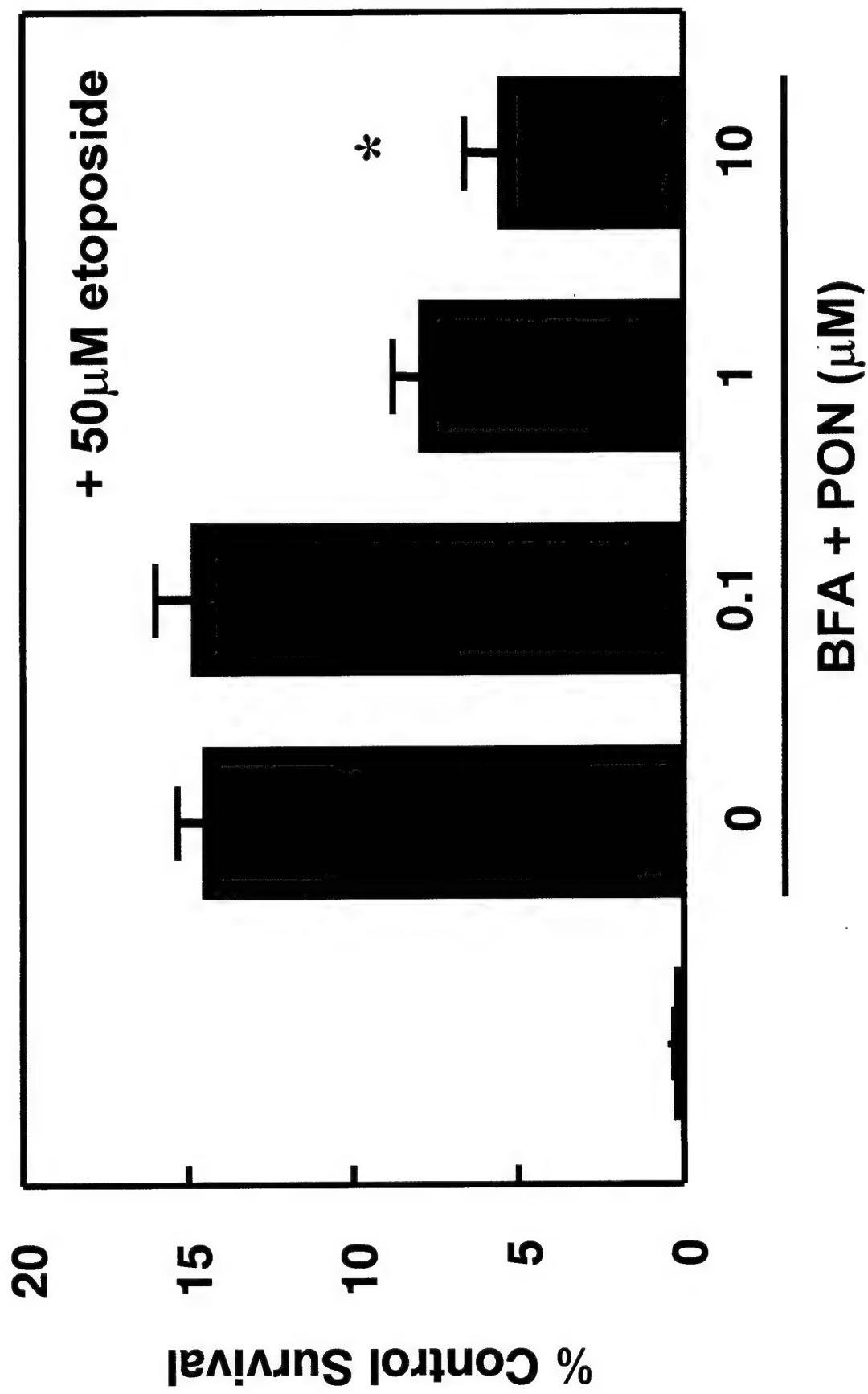
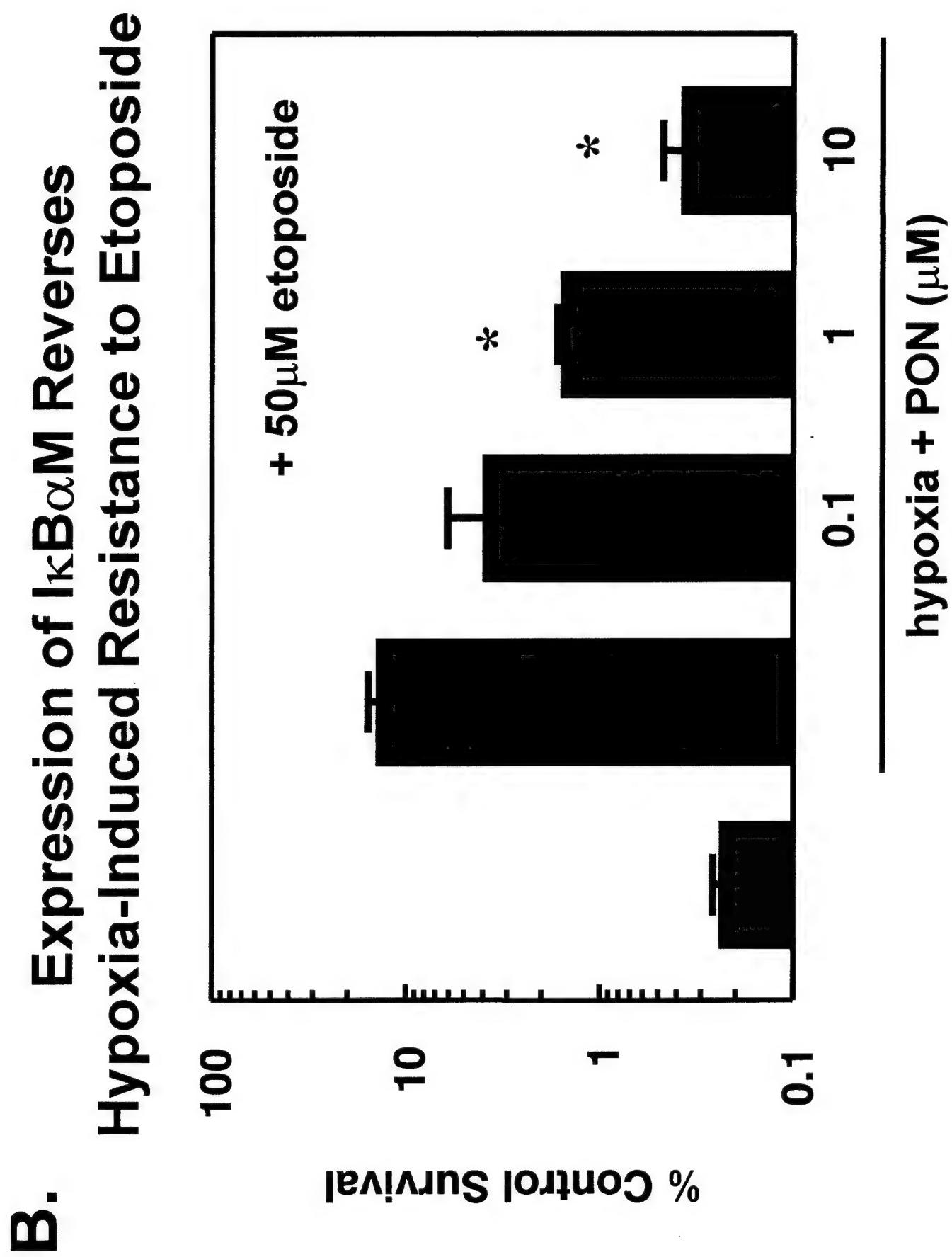
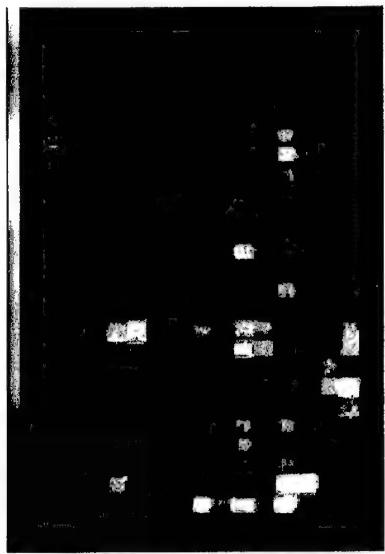
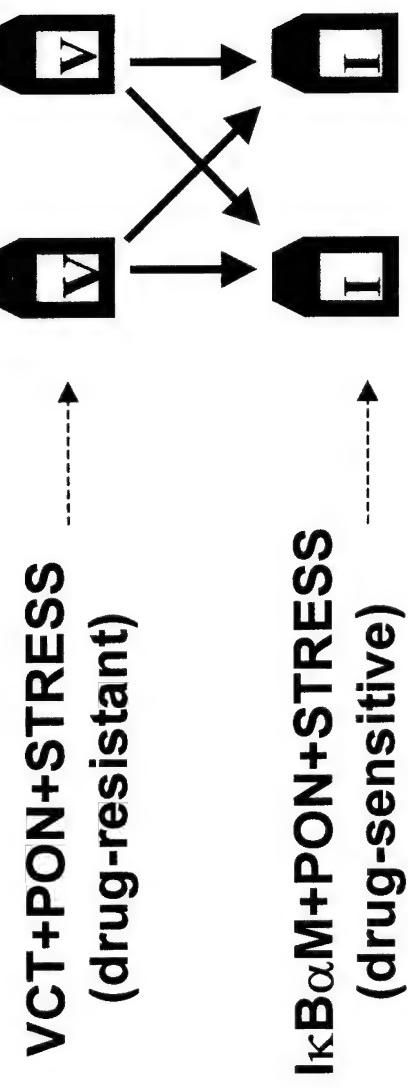


Fig 4B

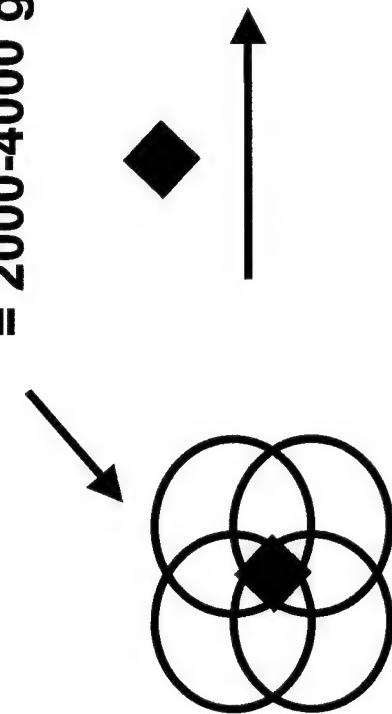


Expression Analysis with the Affymetrix GeneChip®

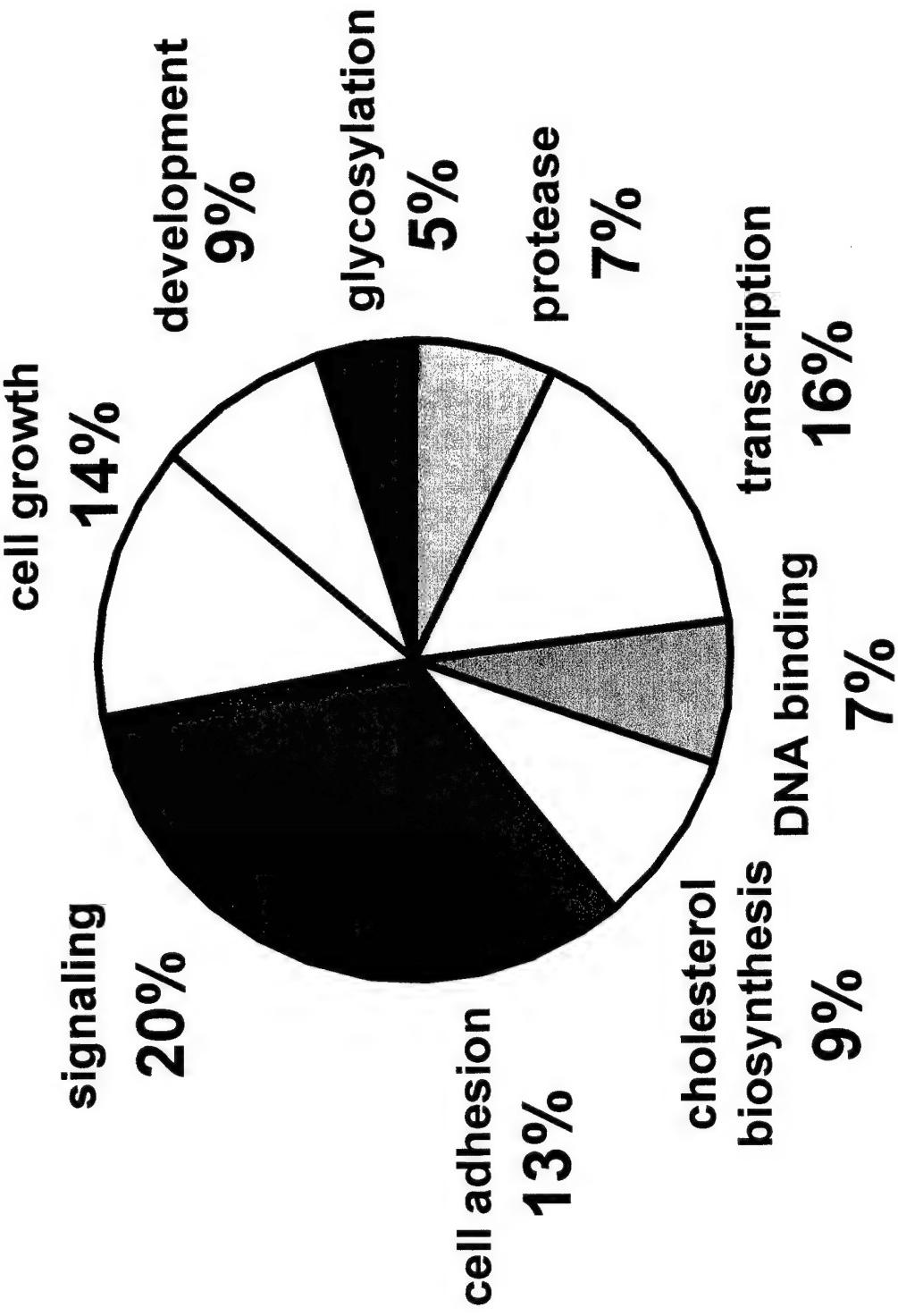


Limit to ≥ 2 -fold change
= 2000-4000 genes

60-100 candidate
resistance-related
genes

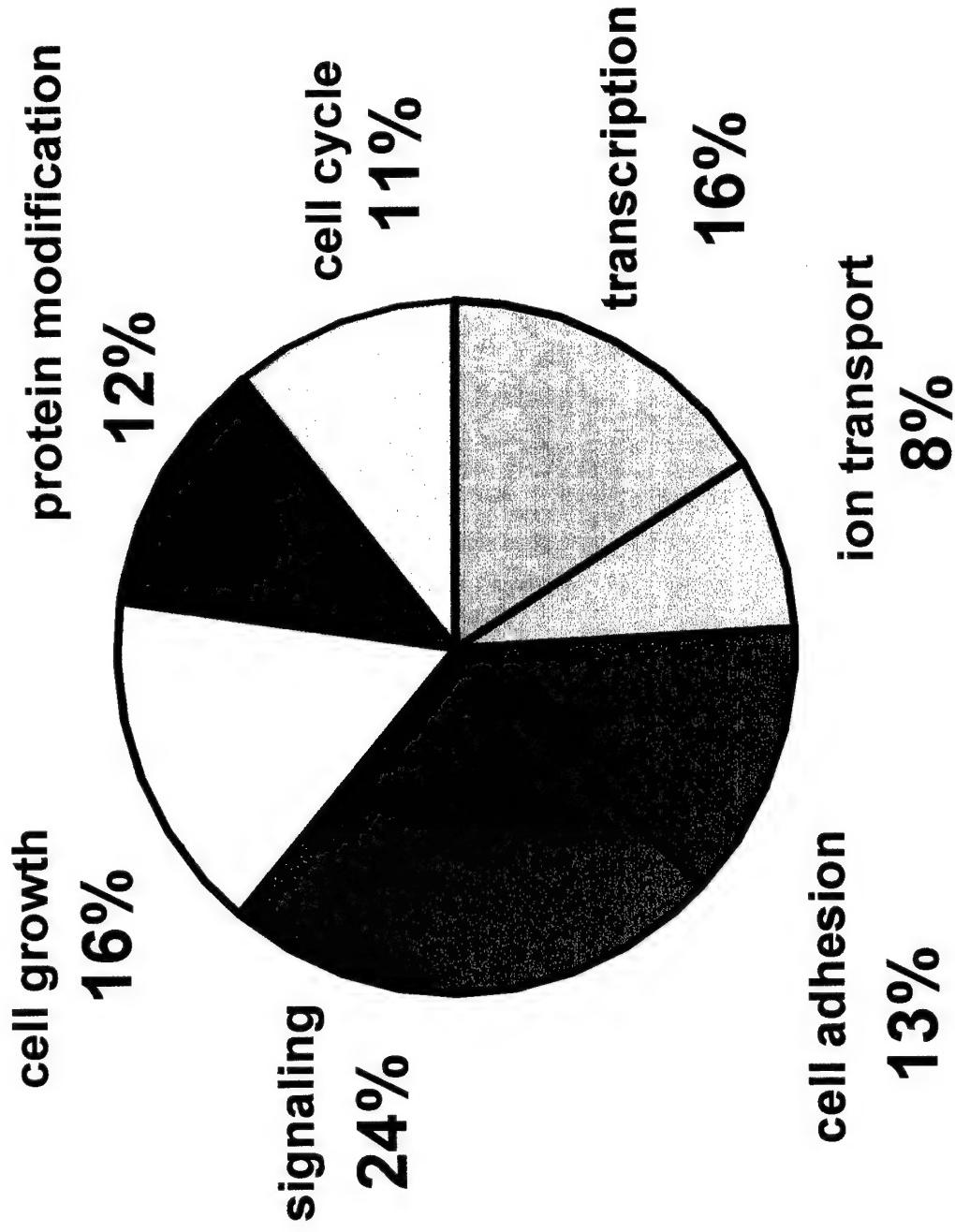


Functional Classifications of Candidate Genes Involved in Both BFA- and HYX-Induced Drug Resistance



Functional Classifications of Candidate Genes Involved in Stress-Induced Drug Resistance

changes in $I\kappa B\alpha M+PON+8hBFA$ compared to $VCT+PON+8hBFA$

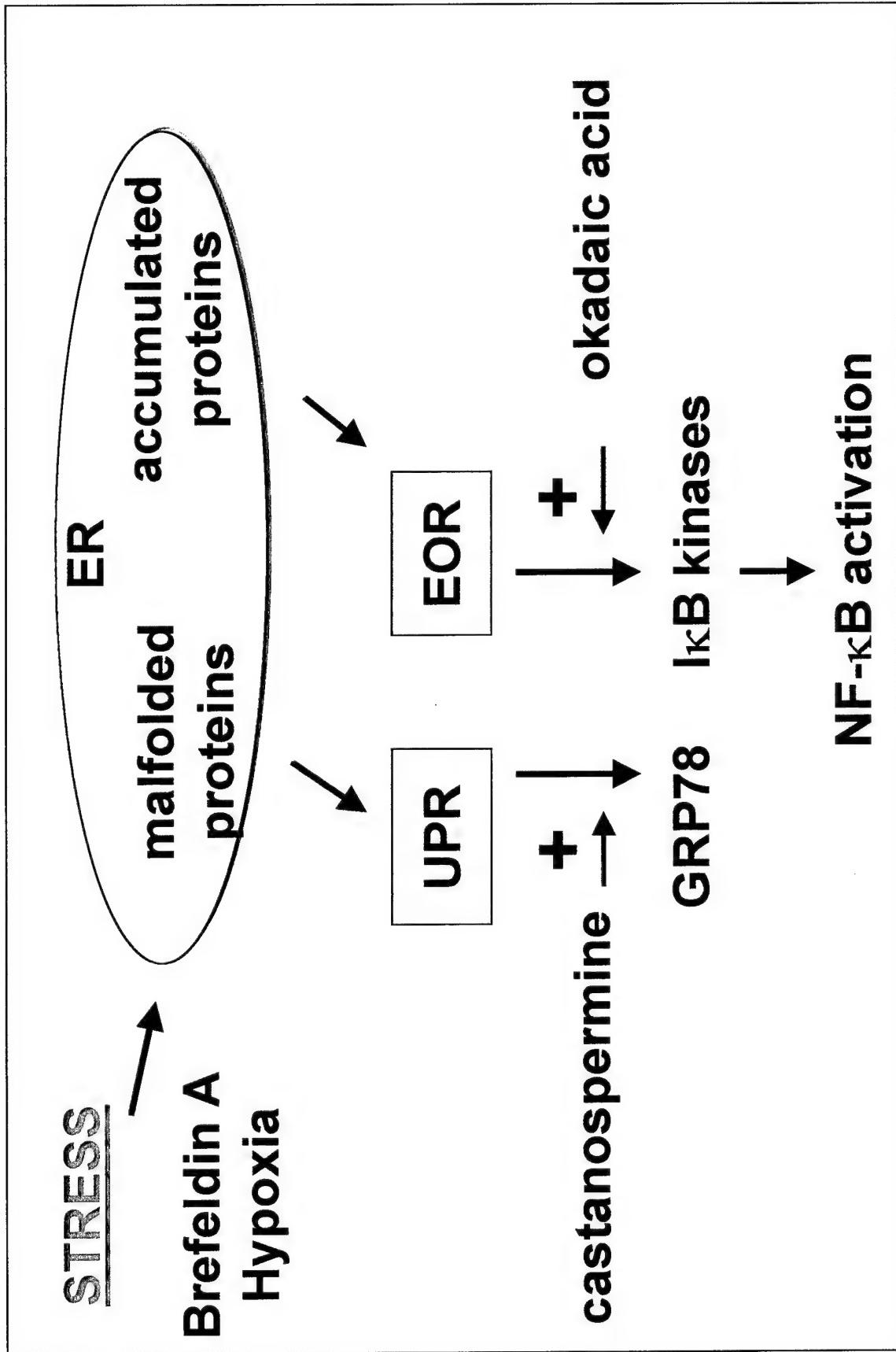


Inducible Expression of the p65 or p50 NF-κB Subunits Causes Resistance to Topoisomerase II Inhibitors and Insensitivity to Stress

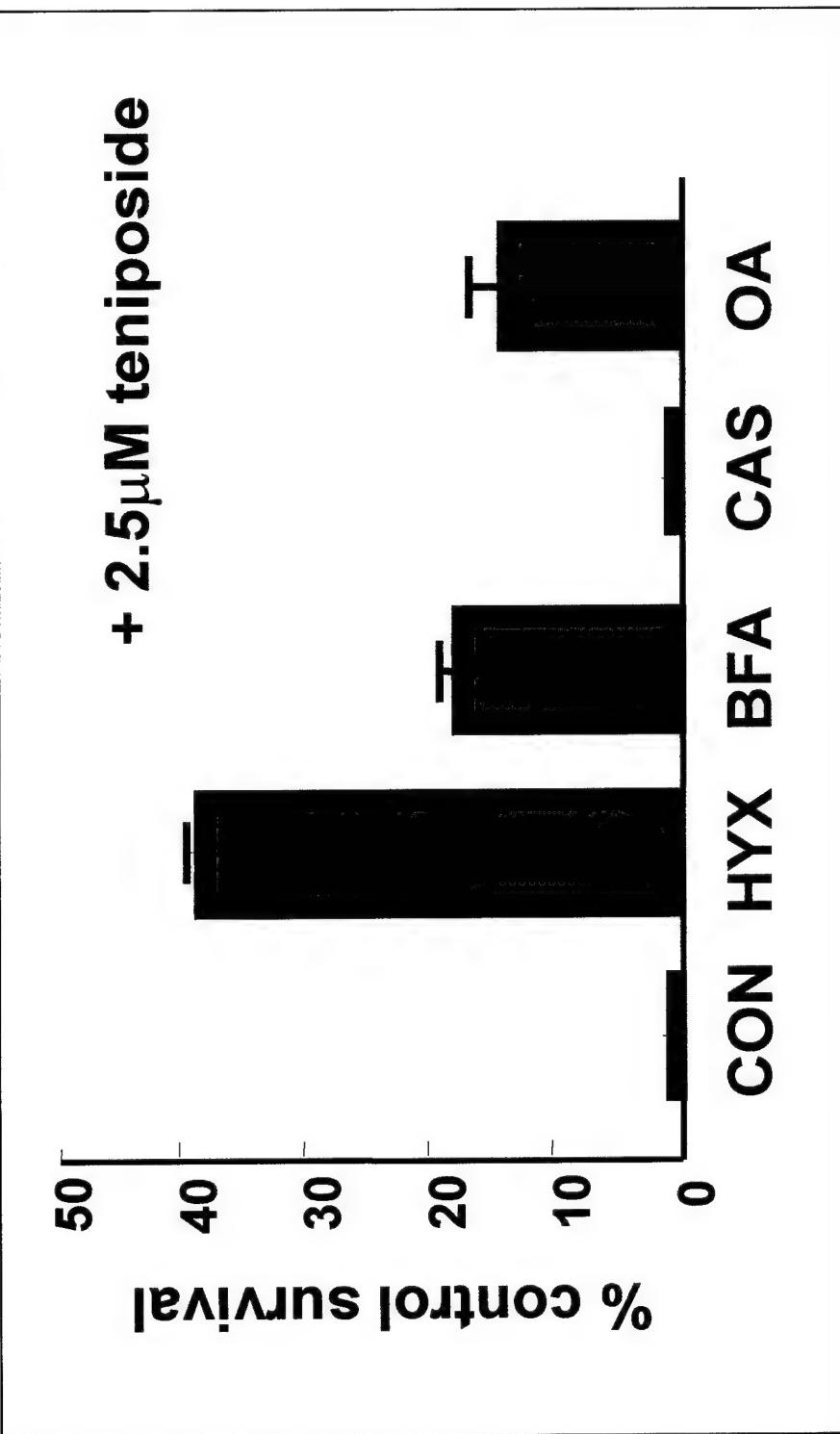
**Lori M. Brandes, Steven R. Patierno, and
Katherine A. Kennedy**

The George Washington University Medical Center
Washington, DC

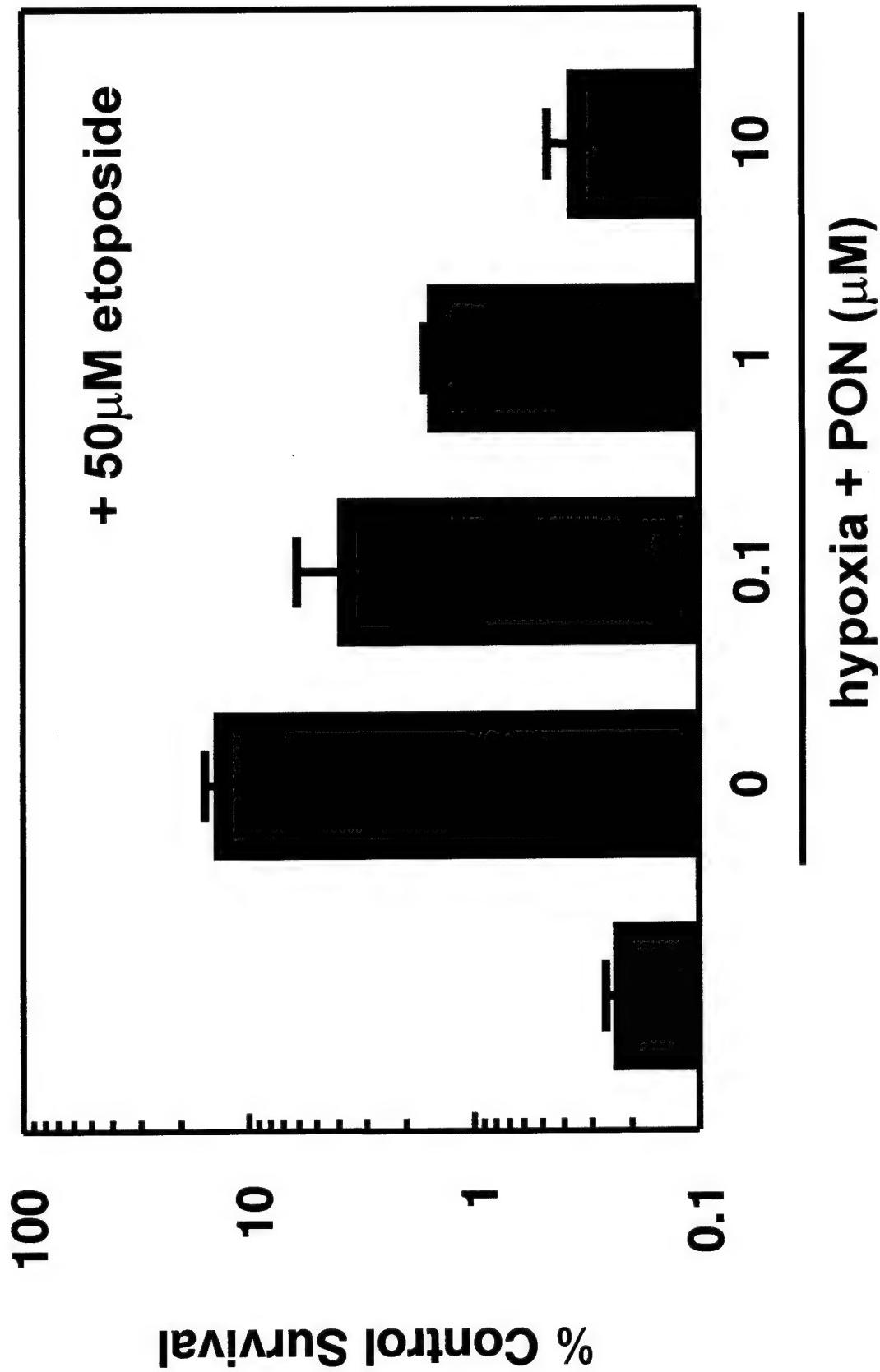
ER-Mediated Stress Pathways



Activation of the EOR Stress Pathway Correlates with Resistance to Teniposide



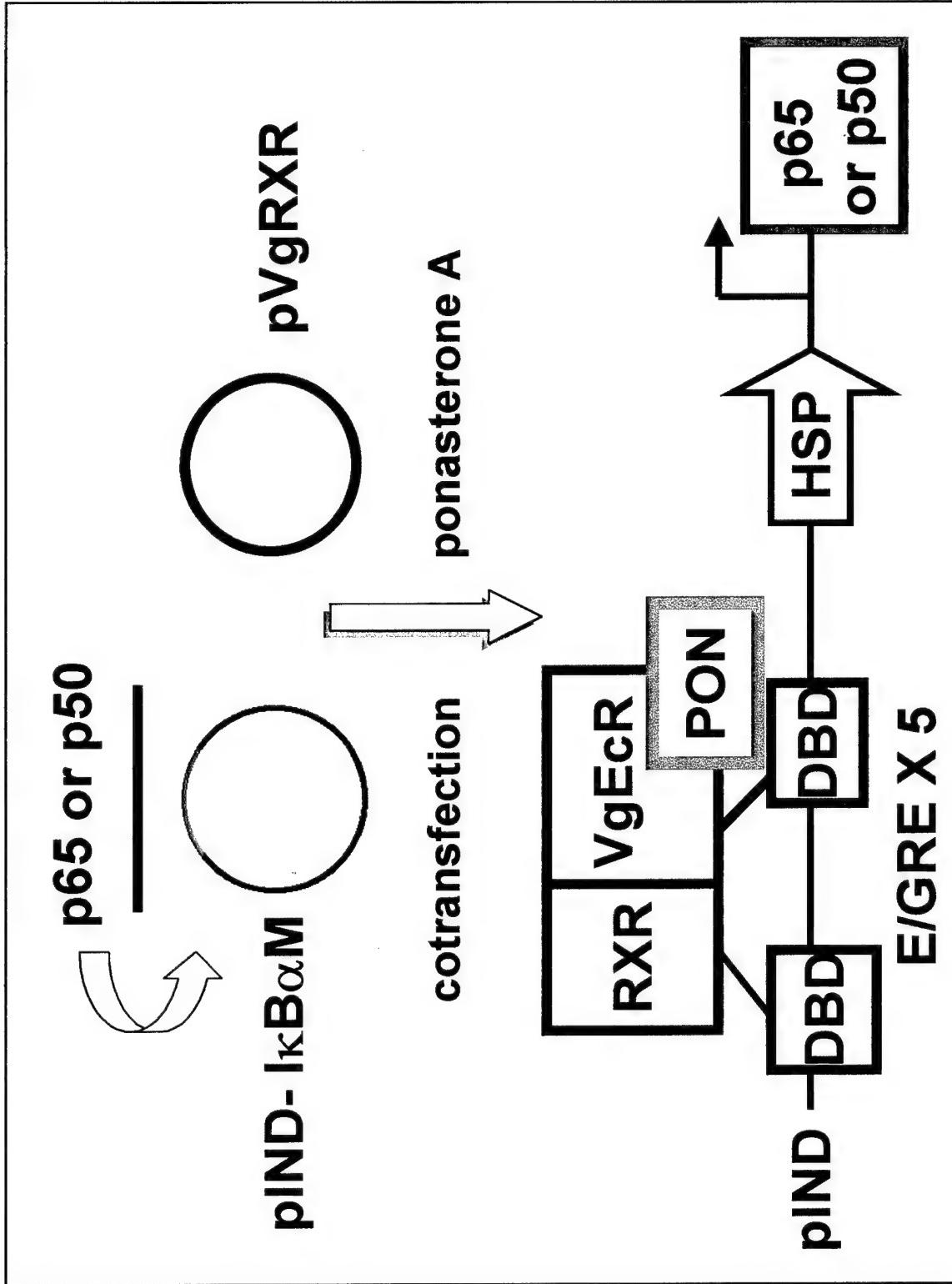
Expression of I κ B α M Reverses Hypoxia-Induced Resistance to Etoposide



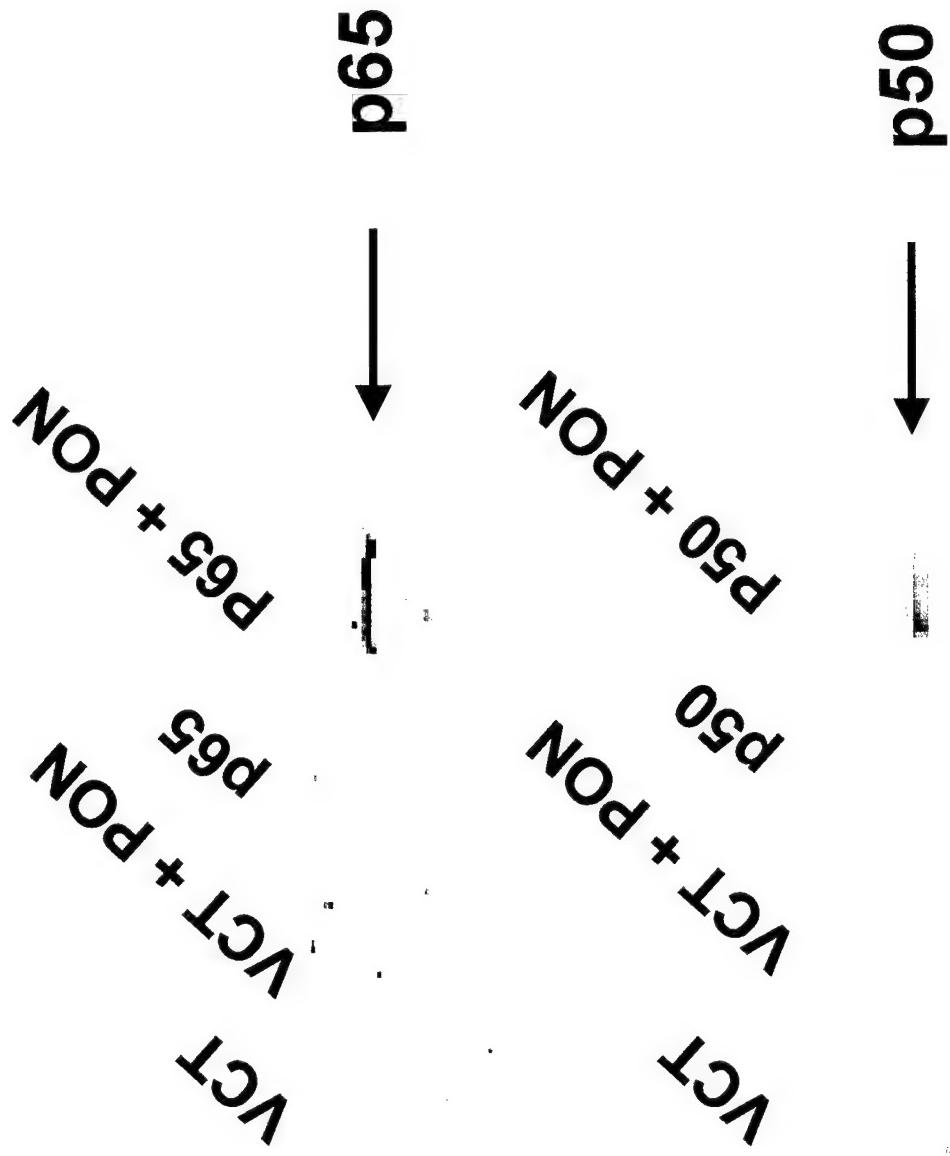
Hypothesis

NF- κ B Activation is Sufficient to Cause
Resistance to Topoisomerase II Inhibitors

The Ecdysone-Inducible Promoter System



Inducible Expression of p65 and p50 in EMT6

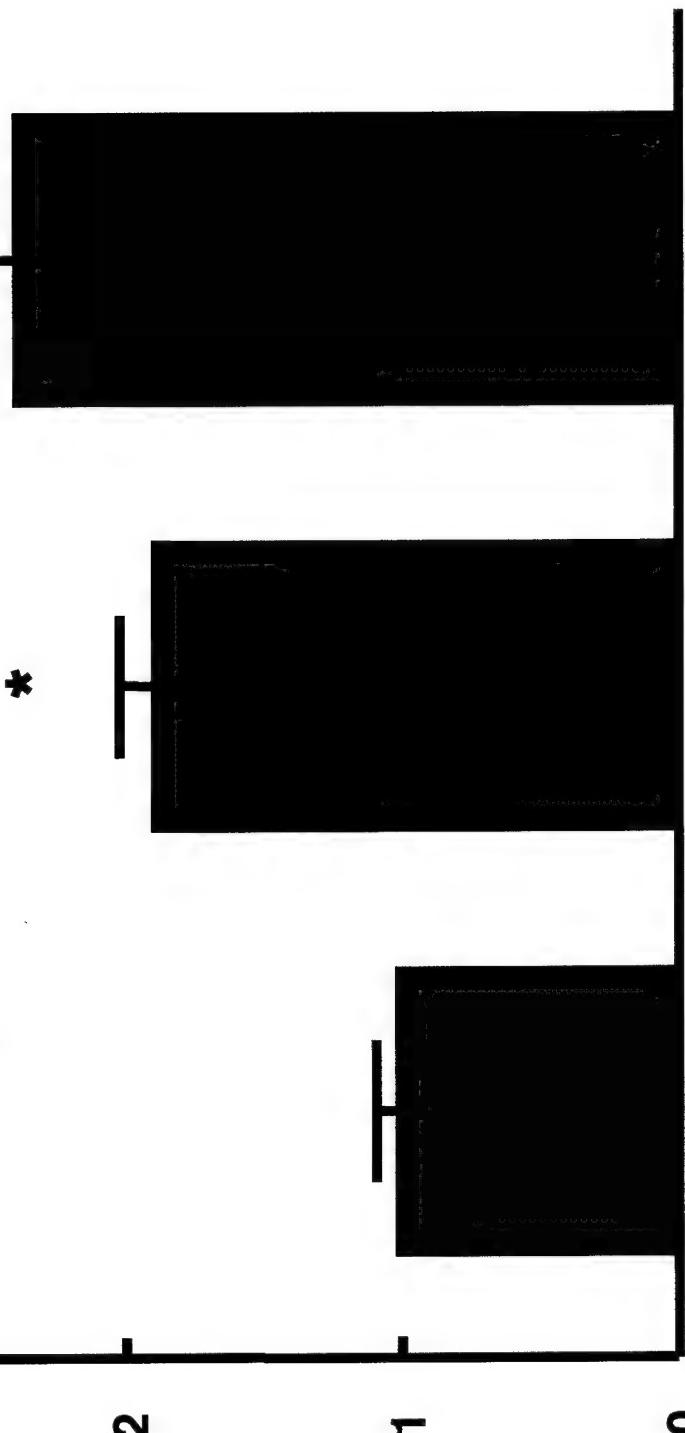


Expression of p65 or p50

Increases NF- κ B Transactivation

+ 10 μ M PON

*



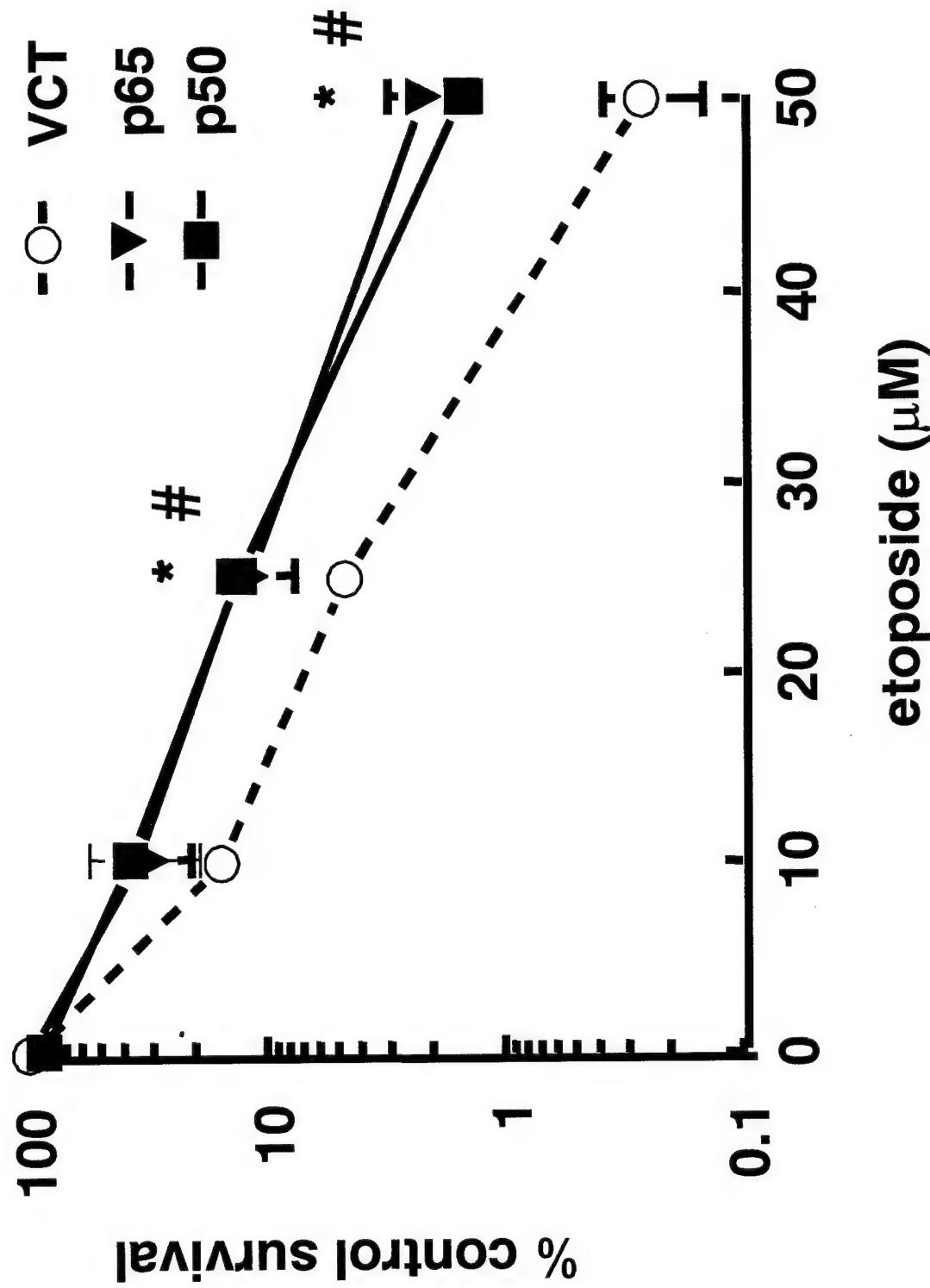
fold control luciferase activity

p50

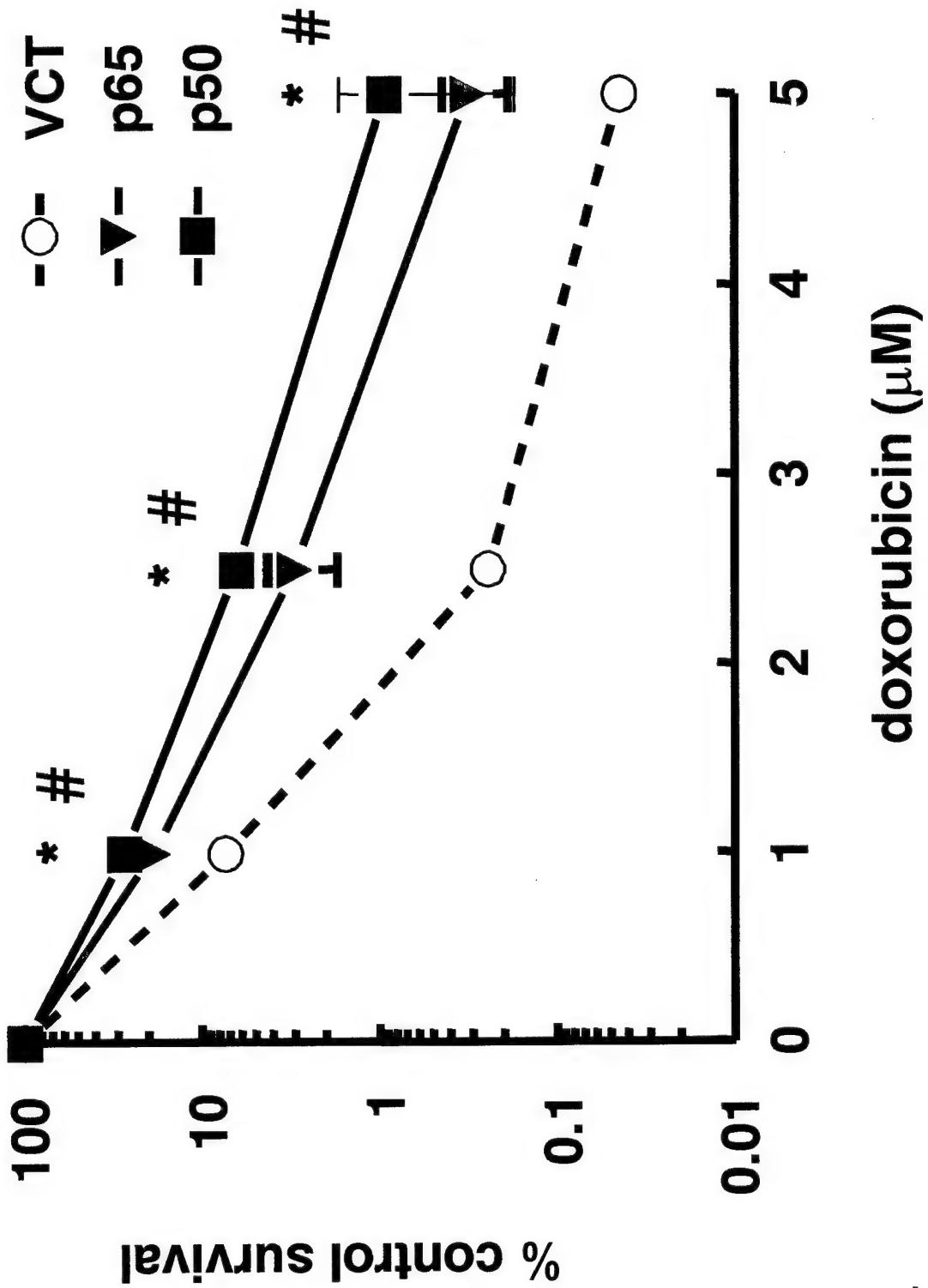
p65

cell line

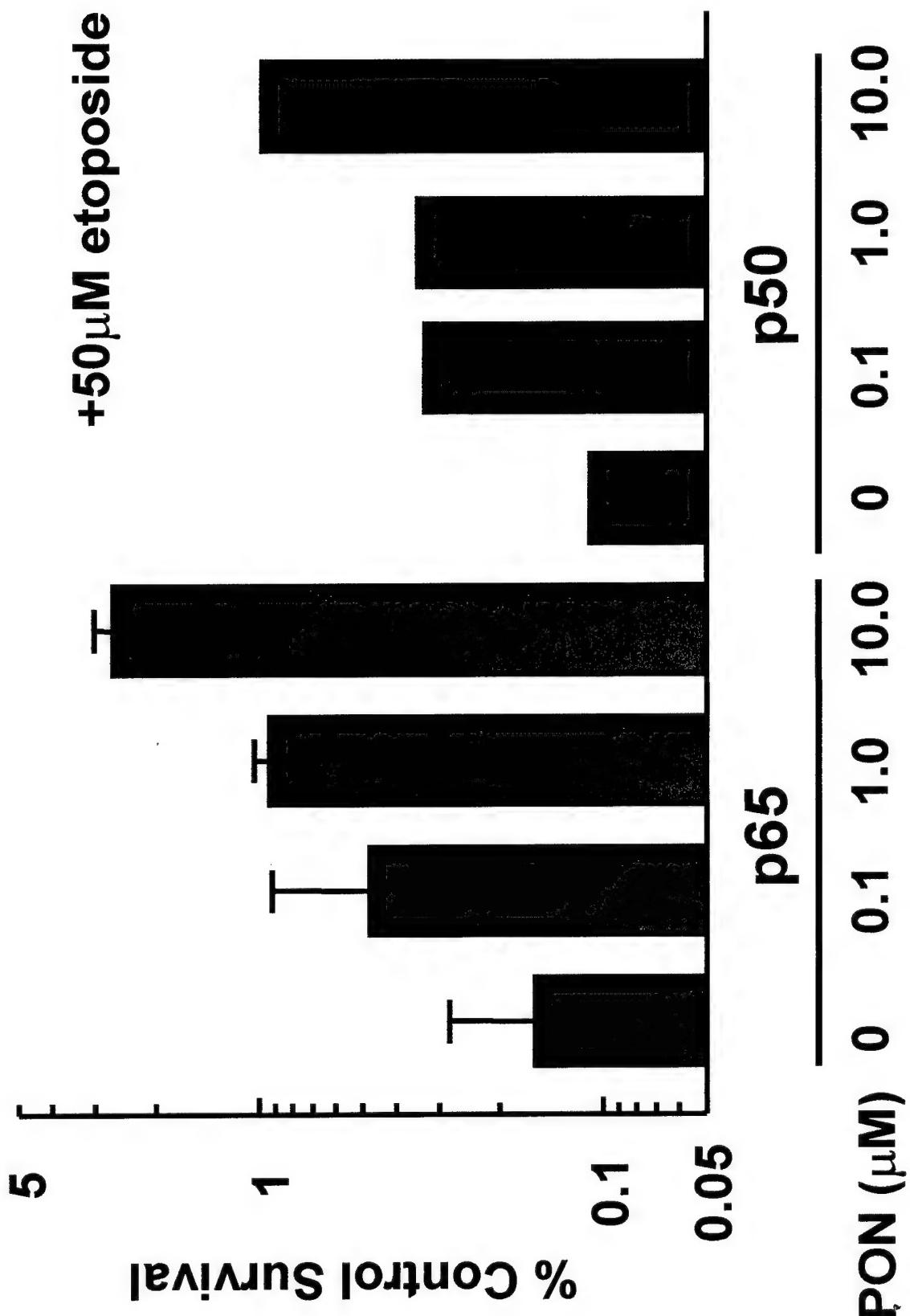
Expression of p65 or p50 Causes Resistance to Etoposide



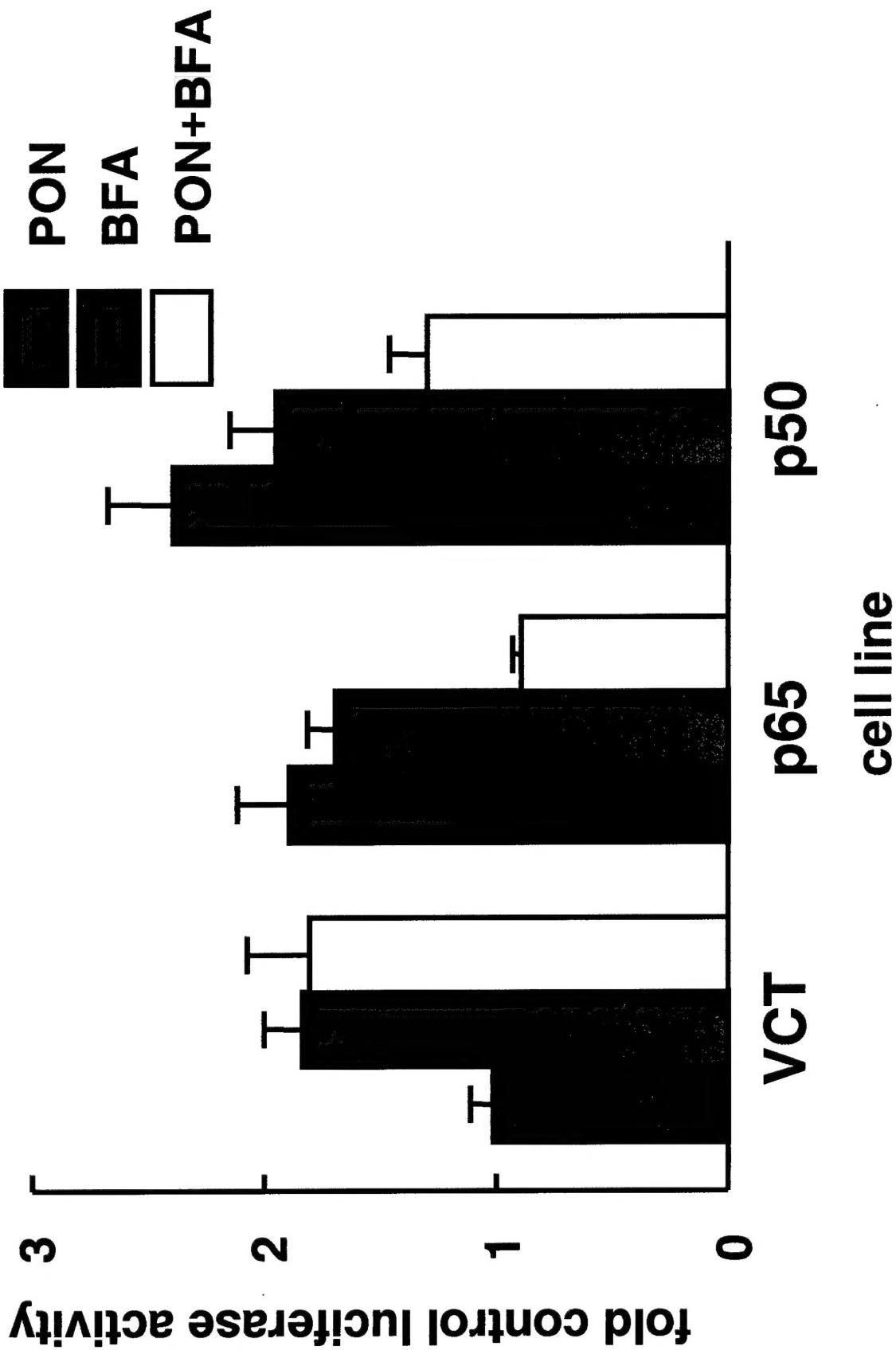
Expression of p65 or p50 Causes Resistance to Doxorubicin



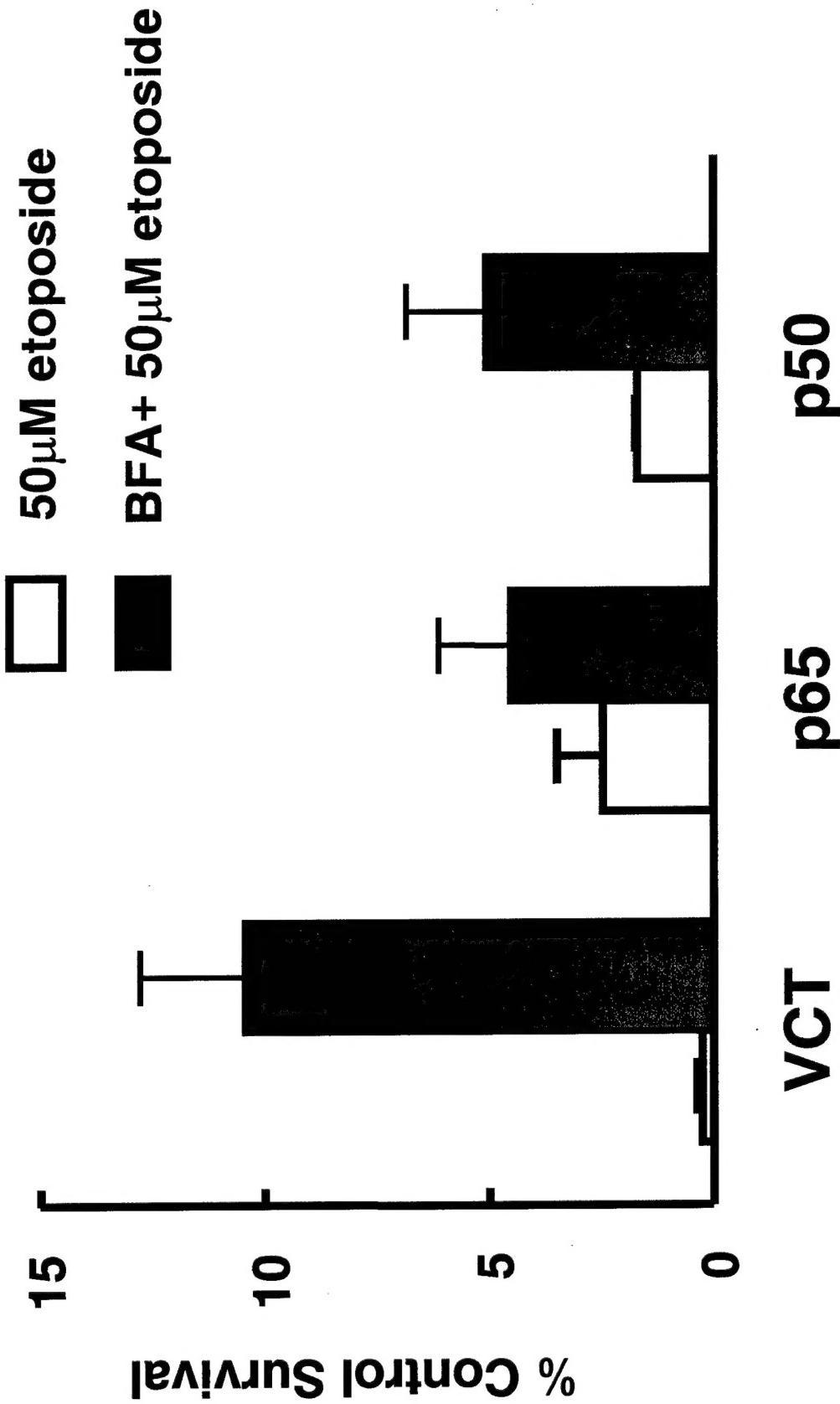
Expression of p65 or p50 Induces Resistance to Etoposide



Expression of p65 or p50 Prevents Stress-Induced NF- κ B Activation



Expression of p65 or p50 Prevents BFA-Induced Resistance to Etoposide



Summary

Inhibition of NF- κ B prevents stress-induced resistance to topoll inhibitors

Expression of p65 or p50 causes:
resistance to topoll inhibitors in the absence of stress
insensitivity to stress-induced NF- κ B activation
insensitivity to stress-induced resistance to
topoll inhibitors

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